

Studies on the β -Lactamase of a
Rhodopseudomonad

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ABSTRACT

Rps.capsulata strains SP108 and SP109 have both been shown to produce a novel type of β -lactamase. Production of β -lactamase by strain SP108 is inducible, a property which correlates with the marked resistance to penicillin exhibited by this strain. Strain SP109, with a non-inducible β -lactamase, does not possess this resistance.

Both strains have been shown to possess identical plasmid complements (four classes of plasmids are present in each) and hybridisation studies have indicated that the β -lactamase structural gene is present on one of these plasmids. Rps.capsulata St. Louis has been found to possess a single plasmid which is distinct from those that exist in strains SP108 and SP109.

The β -lactamase structural gene from strain SP108 has been cloned in both phage and plasmid vectors. The gene was observed to be able to direct the synthesis of β -lactamase when present in the phage system but not when present in the plasmid system.

The cloned fragments have been characterised as a preliminary step to nucleotide sequencing studies.

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ABBREVIATIONS

BSA	bovine serum albumen
cfu	colony forming units
COS(S)	cold osmotic shock (supernatant)
dCTP	deoxycytidine 5'-triphosphate
dNTP	deoxynucleoside 5'-triphosphate
DEP	diethylpyrocarbonate
EDTA	ethylenediaminetetraacetate
EtBr	ethidium bromide
kb	kilo base(s)
Md	mega dalton(s)
PEG	polyethylene glycol
pfu	plaque forming unit
SDS	sodium dodecyl sulphate
TCA	trichloroacetic acid

ANTIBIOTICS

Amp	ampicillin
Carb	carbenicillin
Cm	chloramphenicol
Pen	penicillin
Rif	rifampicin
Strep	streptomycin
Tc	tetracycline
s	sensitive
r	resistant

CHAPTER 1

1.1 The β -lactamases

a. Historical introduction

The β -lactamases are a group of enzymes which hydrolyse the β -lactam bond possessed by the penicillin and cephalosporin groups of antibiotics. This hydrolysis renders the antibiotics biologically inactive. These enzymes are also referred to as penicillinases or cephalosporinases, depending upon which of the above mentioned groups of compounds they hydrolyse preferentially.

The first report of a β -lactamase appeared in the December 28th, 1940, issue of Nature, where Abraham and Chain reported that extracts from certain bacteria destroyed penicillin. There were several reports of β -lactamase producing strains between 1940 and 1960 and although Escherichia coli was included amongst them the Staphylococci were, at this time, the only group of organisms whose β -lactamase posed a serious clinical problem. The two major areas of β -lactamase research during this period were the development of β -lactam antibiotics which were resistant to attack by the Staphylococcal enzyme and the work, of a rather more theoretical interest, carried out by Pollock on the induction of the β -lactamase of Bacillus licheniformis.

The only penicillins in major clinical use prior to 1959 were benzylpenicillin and phenoxymethylpenicillin, and when methicillin was introduced in 1960 to combat the Staphylococcal enzyme it appeared that the clinical relevance of the β -lactamases had completely disappeared.

However, following the introduction, in 1961, of ampicillin it was realised that the β -lactamases may be important in conferring penicillin-resistance in Gram-negative bacteria. This realisation was precipitated by two events. First, the observation that certain cephalosporins, which were developed for clinical use and introduced in 1964, although unaffected by the Staphylococcal enzyme were rapidly destroyed by enzymes from many Gram-negative bacteria. The second was the report by Datta and Kontomichalou (1965) of a resistance to β -lactam antibiotics mediated by R-factors in Gram-negative bacteria, in which this resistance was found to be associated with the production of specific β -lactamases.

In subsequent years β -lactamases from many species of Gram-negative bacteria have been described and several systems of classification have been proposed in order to facilitate identification and assess relatedness of these enzymes. A description of one of these systems will follow a consideration of some of the criteria used in classification. Such criteria include substrate profile, isoelectric point, inducibility, and molecular weight.

b. Criteria used in classification

The substrate profile of an enzyme is a description of its relative hydrolytic activity towards a range of β -lactam substrates. The profile is usually presented as a range of values which indicate the rate at which any given substrate is hydrolysed compared to the rate at

which benzylpenicillin is hydrolysed by the same enzyme preparation. The rate of hydrolysis of benzylpenicillin is designated 100. Thus a profile of benzylpenicillin 100; ampicillin 200; cephaloridine 50 indicates that the enzyme hydrolyses ampicillin at twice the rate and cephaloridine at half the rate at which it hydrolyses benzylpenicillin. One of the advantages of this index is that it is independent of the amount of enzyme being produced by the organism.

Analytical isoelectric focusing permits a direct visual comparison of different β -lactamases as they align themselves at positions corresponding to their isoelectric points (pI) in an electrophoretically produced pH gradient. This technique has been used to distinguish β -lactamases that other biochemical and immunological techniques have failed to differentiate and is sufficiently sensitive to have detected β -lactamase activity in strains where alternative methods have not done so (Matthew et al, 1975). Isoelectric focusing can be carried out on crude enzyme preparations and has been used to screen the β -lactamases of large numbers of bacterial species (Matthew and Harris, 1976).

The majority of Gram-negative β -lactamases that are inducible are chromosomally mediated cephalosporinases, the one documented exception being the β -lactamase of Pseudomonas thomasii 1873E (Sykes and Matthew, 1976). The main application of inducibility, with regard to classification, is therefore the division of the chromosomal cephalosporinases into those enzymes that are

inducible and those that are constitutive (Sykes and Smith, 1979).

One of the earliest reported cases of a non-constitutive Gram-negative β -lactamase was that of the Pseudomonas aeruginosa NCTC 8203 enzyme described by Sabath et al, (1965) and Sykes and Richmond (1971). Induction of this enzyme resembles that of β -galactosidase in the lac operon of E.coli in that enzyme production is proportional to - over a certain range - inducer concentration, and ceases when the inducer is removed (Nordstrom and Sykes, 1974). The two systems differ, however, in that there is a relatively long lag period between addition of inducer and increased β -lactamase production: after addition of 20 μ g/ml benzylpenicillin 80 min elapsed before an increased β -lactamase production was observed (Nordstrom and Sykes, 1974).

As is the case for many enzymes molecular weight estimations have played a role in the characterisation of β -lactamases. These estimations have clearly differentiated enzymes which in other respects are similar, e.g. the β -lactamases encoded by R-factors R-GN238 and R-1818 (R46) (Dale and Smith, 1972). Doubt has, however, been cast on the usefulness of molecular weight estimations due to the widely different values that have been indicated, by a variety of techniques, for a single enzyme (Ambler, 1980), and the fact that β -lactamases with considerably different biochemical properties often have similar molecular weights (Sykes and Smith, 1979).

c. Classification

One of the most recently described classification systems is that of Sykes and Smith (1979). It has a genetic basis, the enzymes being initially grouped according to whether they are chromosomally or R-factor-mediated.

i. Chromosomally mediated enzymes

The chromosomally mediated enzymes are further divided according to whether they are cephalosporinases, penicillinases, or enzymes which exhibit a broad range of substrate specificity.

The first group, the cephalosporinases, is by far the largest of the three and is in turn sub-divided into those enzymes which are inducible and those which are constitutive. Both sub-groups are of approximately the same size and each contain enzymes derived from a variety of bacterial genera.

The chromosomal penicillinases constitute a relatively small group, six enzymes being allocated to it by Sykes and Smith. These authors comment that no good evidence exists for some of the strains, producing β -lactamases placed in these group, not carrying R-factors.

The broad-spectrum β -lactamases do not differ very greatly with regard to substrate profile, but do have easily distinguishable isoelectric points. These enzymes are all constitutive and the majority of them have been isolated from strains of Klebsiella.

ii. Plasmid-mediated enzymes

At the present time there are eleven types of Gram-

negative plasmid-mediated (R-factor-mediated) β -lactamases. These enzymes have been mainly distinguished by substrate profile and isoelectric focusing studies and the majority of them are encoded by several plasmids that are members of different incompatibility groups. Table 1.1 lists the enzymes and the plasmids that have been shown to specify them.

There are two enzymes in the TEM group of β -lactamases, TEM-1 and TEM-2. These enzymes have identical substrate and inhibitory profiles, cross-react immunologically and have similar molecular weights. However, they can be distinguished by isoelectric focusing (TEM-1, pI = 5.4; TEM-2, pI = 5.6, Sykes and Matthew, 1976). The similarity of these enzymes strongly suggests that they are closely related and it is thought that their difference may be the result of a single amino acid substitution.

The OXA group of β -lactamases, so called because of their ability to hydrolyse methicillin and certain isoxazoyl β -lactam substrates, consists of three different types of enzymes, OXA-1, OXA-2 and OXA-3. OXA-2 and OXA-3, with molecular masses of 44,600 and 41,200 respectively, appear to exist as dimers, whereas OXA-1 (M.W. 23,000) is a monomer (Dale and Smith, 1976).

There are four β -lactamases, PSE-1 - PSE-4, which are present on plasmids normally confined to the genus Pseudomonas. Although all these enzymes hydrolyse carbenicillin at least as fast as benzylpenicillin they do not appear to have any outstanding physical similarities

Table 1.1 The types of β -lactamases specified by some Gram-negative plasmids.

TEM-1 (62%)					TEM-2 (15.4%)		SHV-1 (41%)
R497	R401	K90	R978b R963A	R2-72	JR66a-1	R672	p453
R811	R462	K107A	ϕ Amp	pMR0200	S-a-1	R840	R974
R826	R7K	K130	R414b	pMR0360	R64-1	RP9	R1010
R839	R6K	K101	RSU2	RSF0883	R698b	RK2	R1010
R946a	R467	K9	R1033	DW1	R1136b	NC28	R1119
R390	R781	K55A	R893	DW27	PM13	RA01-R2	pUB5451
R394	R784	RY3	R898a	RK1	R1014		R22542
R441	R825	R79	R979	1369E	R906-1		R1239
R471a	R458	pUZ2	R1097	R _{GN14}	RP4		
R494	R934	pUZ3	R938	R1	R40b		
R648	R645	pUZ4	pTM89	RT53	RexProvD		
R771a	RS28	pUZ5	R1143	RT54	R16b		
R398	R _{IP} 69	pUZ9	R1152a	R26	RP1		
R444	R893	pUZ10	R _{IP} 175	N251	R30		
R445	R1097	pUZ11	R1165	R40a	R68		
R446c	R26	pUZ16	RexProvC	R269N	RP636		
R448	RS1030	pUZ21	R934	R447a	R91		
R452	R28K	pUZ23	R837	R447b	R40c		
R454	R963A	pUZ30	R111	R813	RP8		
R461	N168	R26	R1025	R606a	R99		
R692	R527	pMG18	R16a	R935	R746		
RM413	R867S	pMG20	R527	R930	RK2		
R457	RDB1	NTP1	Rms165	R429	RGN823		
R799a		R75	pMG26	R472	NC28		
R787		R959	R2	R707	CR91A		
R459		R870	914	R763	R667		
		R930	R799b	R799a	R84 ²		
		R793a		R820	RPH		

This table was compiled by M. Matthew (personal communication).

The figures in parentheses indicate the frequencies with which particular β -lactamases were observed during the survey of Matthew, 1979 (see text for details).

HMS-1 (0.3%)	OXA-1 (8.8%)	OXA-2 (4.7%)	OXA-3 (1.9%)	PSE-1 (0.6%)	PSE-2 (0.3%)	PSE-3 (0.3%)	PSE-4 (1.7%)
R997	N168	Rte16	R816	RPL 11	R151	Rms149	HL
	R999	R62	R817	Rms139			pMG19
	R1018	R205	R _{IP} 64				
	R1004a	R1135	R55				
	R753	R906-1	R57b				
	R775	Rox166					
	R _{GN} 238	Rox176					
	R455	Rox179					
	R456	Rox 407					
	R609b	R46					
	R656a	R7					
	R755	R16					
	R453	R45					
	RK2	RM98					
		R48					
		RM28					

and thus have only their host genus in common. The genes determining these enzymes have been shown to be transferable to E.coli where they are expressed and confer resistance to the β -lactams as in the pseudomonads (Hedges and Matthew, 1979).

The SHV-1 and HMS-1 enzymes are both determined by plasmids carried by enterobacteria. Their substrate profiles, though different, are both similar to that of the TEM-type β -lactamases, their isoelectric points, however, (SHV-1, pI = 7.6; HMS-1, pI = 5.2) are different to those of the TEM enzymes (Matthew et al, 1979).

Most Gram-negative R-factors that encode a β -lactamase possess a TEM-type enzyme. In a recent survey, Matthew (1979) found that the TEM-1 enzyme was present in 62% of 363 strains which carried plasmid-mediated β -lactamases. The frequencies with which the other Gram-negative plasmid-borne β -lactamases arose in this survey are indicated in Table 1.1.

The plasmid-mediated nature of these enzymes must be, to a large extent, responsible for the wide generic and geographical distribution enjoyed by most of them. In an attempt to explain the particularly wide occurrence of the TEM-1 β -lactamase Hedges et al (1974) suggested that its structural gene may possess special properties which permit it to be translocated from one replicon to another. This view was substantiated by the observation that the amp^r gene (TEM-type β -lactamase structural gene) of RP4 could transpose to the chromosome of the host cell. In turn, Bennett and Richmond (1976), reported transposition

of the chromosomal amp^r to a plasmid, stating that this event was concomitant with an increase in size of the plasmid. Heffron (1975) further observed that transposition of this marker could also occur between two plasmids and, as before, this resulted in a specific size increase for the recipient plasmid. The term transposon was used to describe the element of DNA responsible for the transposition of amp^r and increase in size of the replicon receiving it.

Several transposons have now been identified which encode resistance to, amongst other things, various antibiotics (Calos and Miller, 1980). Transposons have the ability to transfer themselves from replicon to replicon and this type of transfer must have contributed to the overall mobility of the TEM-type β -lactamases, and therefore to their widespread occurrence amongst Gram-negative bacteria.

d. Secretion

In addition to the extensive work that has been carried out on the characterisation of Gram-negative β -lactamases a considerable amount of attention has been paid to several of the Gram-positive enzymes.

The production of β -lactamase by B.licheniformis has been studied to investigate the process by which bacterial cells secrete enzymes. This organism releases about half of its enzyme into the medium, the remainder being cell bound (Pollock, 1961). Yamamoto and Lampen (1976) reported that the membrane-bound β -lactamase

differs from the extracellular form of the enzyme (exopenicillinase) in having a phospholipopeptide of 25 amino acid residues present at its NH_2 -terminus. The amino acid sequence of this extension was determined (Yamamoto and Lampen, 1976) and found to terminate with a phosphatidylserine residue (Yamamoto and Lampen, 1975).

Simons et al (1980) although able to detect a hydrophobic NH_2 -terminal extension of the membrane-bound enzyme (relative to the exopenicillinase) were not able to identify a phospholipid component of it. Furthermore, they isolated a second form of the exopenicillinase, *exoS*. The *exoS* form appears to differ from the normal *exo*-penicillinase, the *exoF* form (whose protein sequence had been previously determined by Meadway (1969)), only in having an extra 8 amino acid residues at its NH_2 -terminus. The sequence of these 8 residues (Simons et al, 1980) did not however correspond to that of the other NH_2 -terminal extension (the phospholipopeptide) presented by Yamamoto and Lampen. DNA sequencing studies have now been completed which confirm the *exoS*-extension amino acid sequence (K. Simons, personal communication).

DNA coding for the *B.licheniformis* β -lactamase generates, in an in vitro transcription-translation system, a penicillinase precursor which is larger than the membrane-bound enzyme. This size difference has been attributed to a further NH_2 -terminal extension and it has been suggested (Simons et al, 1980) that this may correspond to the "signal sequence" initially present on other secreted enzymes (Blobel and Dobberstein, 1975). It is

proposed by Simons et al that this "signal sequence" is removed to expose a new NH₂-terminus that is responsible for anchoring the enzyme to the cytoplasmic membrane. It would appear that the anchoring region may in turn be excised, thus generating the exocellular form of the enzyme.

The DNA sequence, which predicts an initial protein product some 41 residues longer than the exoF exopenicillinase does not contradict this speculation. Indeed, the initial NH₂-terminal amino acid sequence has certain characteristics in common with the signal sequences of other secreted bacterial enzymes (see Osborn and Wu, 1980).

e. Location

Neu and Heppel (1964) stated that certain degradative enzymes were released from E.coli on the formation of protoplasts by an EDTA/lysozyme treatment. This report was followed by the description of another method, the cold osmotic shock procedure, which is also capable of effecting the specific release of these enzymes without impairing cell viability (Neu and Chou, 1967).

This procedure consists of incubation of the cells in a hyperosmolar solution of sucrose and EDTA followed by sedimentation of the cells and exposure to either cold water or a dilute magnesium solution. It has been used to specifically release enzymes from several bacterial genera including: Escherichia, Enterobacter, Shigella, Salmonella, Citrobacter and Serratia. The optimal

procedure varies from species to species depending on such factors as: growth medium, age of culture, and buffer system. Between 15 and 20% of total cellular protein, which produces approximately 15 bands on an SDS polyacrylamide gel, is usually liberated by this procedure (Neu and Chou, 1967)... The enzymes that are released are not extracellular and as they are also released upon sphaeroplast formation (unlike cytoplasmic enzymes) it appears that they may be located at or near the cell surface. The periplasmic space (which exists between the inner and outer membranes) has been proposed as a candidate for the location of these enzymes. The rapid release of the enzymes and electron microscopic evidence support this view.

Neu (1968) reported that the R-factor-mediated β -lactamases of strains of E.coli and Salmonella typhimurium were released by cold osmotic shock, thus indicating a surface location for these enzymes. It was not however possible to release chromosomally mediated β -lactamases by this treatment and it was suggested (Neu, 1968) that this may indicate some intrinsic difference between these two classes of enzymes. (1972) Curtis et al. were able to show that this was not the case by the cold osmotic shock induced release of both types of enzyme.

The cold osmotic shock procedure has been used as the initial stage in the purification of a β -lactamase from S.typhimurium (Neu and Winshell, 1970).

f. Factors affecting resistance to β -lactam antibiotics

The history of the β -lactamases contains a vast number of reports referring to the effects of these enzymes on bacterial resistance to penicillins and cephalosporins. A detailed review of this topic would be too voluminous to be presented here and is unnecessary as the subject has received recent consideration (Richmond and Curtis, 1974; Sykes and Matthew, 1976). However, a brief description of the ways in which β -lactamases have been implicated in resistance to β -lactam antibiotics will be given.

As many β -lactamase genes are carried on transmissible R-factors mechanisms exist by which these genes can be introduced to or eradicated from certain bacterial strains (Richmond and Sykes, 1972; Bouanchaud et al, 1969). It is therefore possible to compare directly the resistance to penicillin of cells with and without an R-factor-mediated β -lactamase. If the β -lactamase gene is chromosomal, but present on a transposon, transfer to an R-factor may be effected which would permit the chromosomal β -lactamase to be subjected to this type of direct comparison.

Sykes and Matthew (1976) compared the resistance of E.coli K12 carrying various R-factors that code for β -lactamase production with the R^- variant. As can be seen from Table 1.2 resistance to the penicillin rises with possession of an R-factor although the increase in resistance to cephaloridine is less marked. Cefuroxime, a cephalosporin that is resistant to the β -lactamases of

Table 1.2 Comparison of single-cell resistance values obtained with an E.coli strain carrying various R-factors and the R⁻ variant (Sykes and Matthew, 1976).

R-factor	Penicillin	Single cell resistance (μg/ml)			Cefuroxime
		Ampicillin	Carbenicillin	Cephaloridine	
R ⁻	16	2	4	1	2
TEM	2500	2500	2500	16	4
RP1	10000	10000	25000	16	4
RGN238	250	250	250	2	4
R57b	31	8	125	4	4

these R-factors, was included as a control to indicate factors other than the β -lactamase which could be responsible for the increased resistance.

Chromosomal β -lactamases have been shown to contribute to the resistance of certain strains by the isolation of sensitive mutants which seem to differ from the wild-type only in having lost β -lactamase activity (Sykes and Matthew, 1976). The isolation of non-inducible mutants from bacterial strains whose β -lactamase is normally inducible have also illustrated the role of the enzyme in effecting resistance (Rosselet and Zimmerman, 1973).

Strains which synthesise increased amounts of β -lactamase, because of the possession of an increased number of β -lactamase genes, have been found to be more resistant to β -lactams. This phenomenon has been observed for both R-factor and chromosomally determined enzymes. Uhlin and Nordstrom (1977) reported that for the copy-number mutant plasmid R1 drd-19 the amount of β -lactamase and resistance to penicillin were both proportional to copy-number of the plasmid.

The chromosomal β -lactamase gene of E.coli is present in a single copy and does not contribute to penicillin resistance in laboratory experiments. Normark et al (1977), however, isolated a series of mutants that had an increased resistance to penicillin which was correlated with an increased β -lactamase production. This series of mutants was found to be due to multiplication of the segment of the DNA carrying the β -lactamase gene.

The other major factor concerned with resistance of

Gram-negative bacteria to the β -lactams is the presence of a barrier which restricts access of these antibiotics to their target sites within the cell.

The cell envelope of Gram-negative bacteria consists of three distinct layers; the inner (cytoplasmic) membrane, the murein (peptidoglycan) layer, and the outer (lipoprotein/lipopolysaccharide) membrane. It is the outer membrane component that acts as a diffusion barrier limiting passage of molecules into and out of the cell. The molecular-sieving properties of this membrane are due to the presence of pores, and it is believed that a protein component of this membrane is responsible for the formation of these membrane channels. Mutants of S.typhimurium that lacked the "matrix protein" were deficient in diffusion of the β -lactam cephaloridine across the outer membrane, and were also less able to utilise low concentrations of several low molecular weight metabolites (Wickner, 1979)

Evidence connecting the outer membrane diffusion barrier with β -lactam resistance comes from two main sources. Firstly EDTA, which has been reported to disrupt the outer membrane (Leive, 1965), increased the sensitivity of various Gram-negative bacteria to penicillins and cephalosporins (Hamilton-Miller, 1965). Secondly, the studies of cell wall mutants, which are not altered in their ability to synthesise β -lactamase, have shown increased sensitivity to β -lactam antibiotics. E.coli cell wall mutants showed increased sensitivity to penicillins but little or no increase in sensitivity for

the cephalosporin tested. Ps.aeruginosa mutants, however, were more sensitive to both classes of compounds (Sykes and Matthew, 1976). These studies indicate that barriers limiting diffusion of β -lactams into the bacterial cell do exist, but that they may be species specific.

As all Gram-negative bacteria possess outer membranes and many also possess β -lactamase activity it is likely that resistance to the β -lactams is, in the majority of cases, not simply due to one of these factors but to the concerted action of both. A method of expressing the interaction of these factors has been described and is referred to as "crypticity". Crypticity is the ratio of β -lactamase activity in broken cells to the β -lactamase activity in intact cells (Richmond and Curtis, 1974). Crypticity values are determined by incubation of a given substrate with a preparation of washed cells both intact and after sonication. Crypticity is thus a function of the "barrier" effect of the outer membrane and the kinetics of the enzyme. A high crypticity value indicates the presence of a permeability barrier although a low value does not exclude this possibility.

Resistance to several types of antibiotics has been shown to be due to a decrease in the affinity of the targets of lethality for the antibiotics. Spratt (1978) reported the first case of this type of resistance to a penicillin.

The cytoplasmic membrane of E.coli possesses

seven proteins which bind $[^{14}\text{C}]$ - benzylpenicillin, and are known as penicillin-binding proteins (PBPs). Certain of these PBPs have been identified as the targets with which β -lactam antibiotics interact to produce their lethal effects (Spratt, 1975).

The penicillin mecillinam binds only to PBP2 of E.coli. Spratt was able to isolate a mutant E.coli strain which grew normally, but with increased resistance to mecillinam, at 30°C and abnormally at 42°C. This mutant was found to have a thermolabile PBP2 which exhibited altered mecillinam binding at 30°C and did not bind the penicillin at all at 42°C. Therefore, resistance to mecillinam appears to be due to a thermolabile PBP2 which has altered mecillinam binding properties.

It is thought unlikely that this type of resistance is common in nature as β -lactams usually bind to more than one PBP. Furthermore, high resistance would depend on the virtual exclusion of the β -lactam from the active site of the PBP without impairing binding of the normal substrate (Spratt, 1978).

g. Sequence information

The ultimate classification system for any group of proteins must be one based upon the primary structure of these molecules. However, as sequence information is difficult to obtain, for even a small number of proteins, such a system for the β -lactamases is, as yet, impractical. Five different β -lactamases have been completely sequenced and though these sequences undoubtedly constitute only a

small proportion of those that exist interesting information regarding the origin, evolution, classification, and other structural aspects of these enzymes has been derived from them.

The first four β -lactamase sequences were determined by work carried out in a single laboratory. These were the sequences of the S.aureus PC1 (Ambler, 1975); B.licheniformis 749/C (Meadway, 1969); B.cereus 569/H (Thatcher, 1975); and TEM (Ambler and Scott, 1978; Sutcliffe, 1978) enzymes. The enzymes exhibit differences in isoelectric points, substrate profiles, inducibility, and kinetic parameters. They do, however, have approximately equivalent molecular masses and possess a certain amount of sequence homology. In fact, this homology is so extensive that it has been suggested to be most reasonably explained by divergence from a single ancestral gene (Ambler, 1980). They are all regarded as being class A enzymes in the structurally based classification system proposed by Ambler (1980).

At the present time there is only one member of class B of this system, the B.cereus β -lactamase II (Abraham and Waley, 1979). The sequence determination of this protein is still in progress but it does not appear to share any homology with the proteins from class A (R.P. Ambler, personal communication). Furthermore, the B.cereus β -lactamase II is the only β -lactamase known to have a metal (Zn) cofactor requirement. These observations have led Ambler (1980) to suggest that the β -lactamases have a polyphyletic origin.

The fifth β -lactamase whose sequence has been determined is the ampC E.coli K-12 chromosomally mediated enzyme (Jaurin and Grundström, 1981). This enzyme is a cephalosporinase and has a molecular mass of 39,600. There is a suggestion that the ampC enzyme has blocks of sequences which show homology with regions of the consensus sequence of the class A enzymes. One of these regions contains the serine-70 residue (Ambler, 1979) which is included amongst those residues which occupy the same site in all class A enzymes. Certain evidence suggests that serine-70 may form part of the active site of these enzymes (Cartwright and Coulson, 1979).

The blocks of supposedly homologous sequence in the ampC enzyme are situated at the COOH-terminus whilst the sequences to which they correspond in the class A enzymes are present at the NH₂-terminus. Thus, although the ampC and class A enzymes may have evolved from a common ancestor it is considered appropriate for this cephalosporinase to become the definitive member of class C. Other Gram-negative chromosomally mediated enzymes may eventually be placed in the same class, e.g. the inducible β -lactamase of Ps.aeruginosa (Sabath et al, 1965).

As further studies are carried out on other β -lactamases it is likely that classes will be added to this system. At the present time the most probable candidates for these alternative classes are the β -lactamases specified by the R-factors R151 and Rms149 (PSE-2 and PSE-3 respectively). These enzymes have relatively small molecular masses (12,000) (Matthew, 1978).

The large number and diversity of the β -lactamases have resulted in their achieving a certain amount of theoretical importance, besides the clinical importance which is of a rather more practical nature.

The theoretical importance arises from the use of certain β -lactamases in various types of enzymological studies. As stated above, certain of these studies have been, to a great extent, dependent upon sequence information and therefore the extension of them and development of new ones would be aided by further sequence information. This is one of several reasons for the study described in this work.

1.2 The Rhodopseudomonads

a. General introduction

The purple nonsulphur bacteria (Rhodospirillaceae) are the best studied and most diverse group of photosynthetic bacteria. Although most grow optimally as anaerobic photoorganotrophs they also have the ability to adopt alternative modes of growth. The family Rhodospirillaceae is comprised of 4 genera of which the genus Rhodopseudomonas is the most diverse (Pfennig, 1977).

It has been suggested that species of the genus Rhodopseudomonas fall into three different groups, depending upon morphological considerations (Pfennig, 1977). Two members of species group 1 Rhodopseudomonas capsulata and Rhodopseudomonas sphaeroides have recently received increased attention, certain aspects of which, relevant to the work presented here, will now be considered.

b. The gene transfer agent

The first system of genetic exchange observed in a photosynthetic bacterium was that reported to occur in Rps.capsulata (Marrs, 1974). Mixed growth of two strains of Rps.capsulata, one resistant to rifampicin and the other resistant to streptomycin, produced doubly resistant cells at a much higher frequency than the corresponding control situations. Thus suggesting that the Rif^r, Strep^r phenotype was due to the interstrain transfer of one or both of the resistances. The transfer was mediated by a DNAase and RNAase resistant, filtrable agent termed the gene transfer agent (GTA), which was capable of transferring all genetic markers with which it was tested. Wall/^{et al}(1975) examined the ability of thirty-three independently isolated strains of Rps.capsulata to produce and receive GTA. She found that half of the strains could participate in both directions of GTA genetic exchange whilst a limited number of strains could either; only donate, only receive, or neither donate nor receive. Furthermore, Wall/^{et al}(1975) observed that GTA mediated gene transfer was restricted to Rps.capsulata, other rhodopseudomonads being incapable of producing or receiving GTA. This indicates a high degree of species specificity for this system.

GTAs are normally produced in low yields and this has made their characterisation difficult, however, Yen et al (1979) were able to isolate a mutant which exhibited an increased production of GTA. Studies with this "overproducer" strain have permitted the morphological

and macromolecular characterisation of GTA. The morphology is essentially that of a small bacteriophage with an icosahedral head which is joined, by a collar, to a tail of variable length - five distinct species of protein have been isolated from GTA. The nucleic acid of this agent is double-stranded, linear DNA of molecular mass 3.6×10^6 (Solioz and Marrs, 1977). Furthermore, it is found, by restriction endonuclease analysis, to be equal in complexity to Rps.capsulata total DNA, suggesting that no more than a small proportion of GTA particles may contain phage-like DNA (Yen et al, 1979). It should be noted that it was not possible to demonstrate plaque-formation by GTA although this was attempted using several strains of Rps.capsulata as indicators (Wall/^{et al} 1975).

c. Rps.capsulata.

Weaver et al (1975) studied thirty-three independently isolated strains of Rps.capsulata with the intention of providing a more comprehensive characterisation of the species. The authors concluded from studies on the morphology, physiology and nutritional properties of these strains that members of this species form a stringent taxonomic grouping. Two properties emerged from this study which it was thought would be useful in distinguishing Rps.capsulata strains from other rhodopseudomonads. First, all strains exhibited an unusual sensitivity to penicillin, far greater than that shown by closely related species. Secondly, all strains examined were susceptible to lysis by one or more strains of species-specific bacteriophage.

The bacteriophages were initially isolated by Wall et al (1975) in order to examine possible relations between GTA and bacteriophage. The ninety-five phage originally isolated were found, by host-range studies, to represent sixteen different virus types. The phage formed plaques of various types of morphology and were all able to lyse cells grown either photosynthetically or aerobically in the dark. None of the phage, however, were capable of forming plaques on members of the Rhodospirillaceae, other than Rps.capsulata, or mediating genetic transduction. Furthermore, there appeared to be no correlation between capability of strains to donate or receive GTA and susceptibility to bacteriophage.

d. Plasmids

Few reports have been made describing plasmids which occur naturally in the Rhodospirillaceae. Gibson and Niederman (1970) described the isolation, from Rps.sphaeroides NClB 8327, of two large plasmids of a similar size (70-75 Md), but with slightly different base compositions. Saunders et al (1976) were, however, able to isolate three plasmids, of sizes 75, 66 and 28 Md, from Rps.sphaeroides 2.4.1 and they suggested that the two larger plasmids may correspond to those isolated by Gibson and Niederman.

Photosynthetically incompetent (Pho^-) strains were isolated from Rps.sphaeroides 2.4.1 after incubation with sodium lauryl sulphate (strain SLS 1) or exposure to N-methyl-N-nitro-N' nitrosoguanidine (strain V-2)

(Saunders et al, 1976). The known curing capabilities of sodium lauryl sulphate and inability of strain SLS 1 to revert suggested that the Pho^- phenotype may be due to loss of a plasmid. However, an electron microscopic analysis of the plasmids from this strain revealed that three classes of plasmids still existed although their sizes were now 75, 66 and 34 Md. It is not possible to infer, from the data presented, a direct relationship between the observed size change of the third class of plasmid and the Pho^- phenotype. Strain V-2, which had a plasmid complement identical to that of the parental strain, was able to undergo reversion to Pho^+ at a frequency of between 10^{-7} and 10^{-8} per cell division. Attempts to transfer genetic markers present in strains 2.4.1 and SLS 1, to other strains of Rps.sphaeroides, by means of conjugation were not successful (Saunders et al, 1976).

Rps.capsulata BH9 has also been found to possess two classes of plasmids. The sizes of the plasmids are 94 and 74 Md, and as with the Rps.sphaeroides plasmids no genetic functions have yet been attributed to them. The possibility exists that the 74 Md plasmid of Rps.capsulata corresponds to the 75 Md plasmid of Rps.sphaeroides, however, no studies have yet been carried out to demonstrate any homology between them.

Although no conjugative abilities have been demonstrated for the plasmids native to Rps.sphaeroides, plasmids from other Gram-negative bacteria can exhibit such properties when present in this species (Sistrom, 1977; Miller and Caplan, 1978). Sistrom (1977) was

able to transfer R68.45 from Ps.aeruginosa PAO25 to Rps.sphaeroides WS23 and Rhodopseudomonas gelatinosa 2.2.1. The R-factor was noted to confer neomycin and carbenicillin resistance to the Rps.gelatinosa strain but only neomycin resistance to Rps.sphaeroides WS23. The carbenicillin sensitivity of Rps.sphaeroides WS23 (R68.45) correlates an absence of β -lactamase activity in this strain (a TEM-type β -lactamase is encoded by R68.45), whilst Rps.gelatinosa 2.2.1 (R68.45) does possess this activity. Furthermore, Rps.sphaeroides WS23 (R68.45) is capable of transferring neomycin and carbenicillin resistance to Rps.gelatinosa but only transfers neomycin resistance to other strains of Rps.sphaeroides.

Similar observations have been made with RP4 which was transferred from E.coli to Rps.sphaeroides 2.4.1 (Miller and Kaplan, 1978). In the E.coli host the plasmid conferred resistance to kanamycin, tetracycline and carbenicillin, whereas when present in Rps.sphaeroides only the first two of these resistances were exhibited. However, as with R68.45, it appears that loss of the Carb^r phenotype is not due to the loss of genetic material as when RP4 is transferred from Rps.sphaeroides to an E.coli strain all three resistances are once more expressed. RP4 has been transferred from Rps.sphaeroides CU604 (RP4) to Rps.capsulata SB1003 (Miller and Kaplan, 1978) and R68.45 has also been transferred into Rps.capsulata (J.D. Wall, unpublished observations). In the latter case carbenicillin resistance was not observed.

e. Isolation of a penicillin-resistant strain of
Rps.capsulata.

As previously stated strains of Rps.capsulata exhibit a characteristic sensitivity to penicillin. However, Weaver et al (1975) isolated a strain of Rps.capsulata, SP108, which had a marked resistance to this antibiotic. Further studies showed that this strain, unlike other Rps.capsulata strains tested, produced a β -lactamase and it is therefore likely that this is the cause of the Pen^r phenotype of strain SP108.

J.D. Wall (personal communication) obtained what was originally thought to be a spontaneously occurring Pen^s isolate of SP108, this was named SP109. However, the possibility does exist that strain SP109 was present as a contaminant in the initial isolation of strain SP108.

This thesis describes studies on the β -lactamase and Pen^r of strain SP108.

Rps.capsulata St. Louis (Weaver et al, 1975) is a wild-type strain which exhibits the usual penicillin-sensitivity of this species. It has been used as a "control" strain in certain parts of the study described herein.

CHAPTER 2

2.1 Growth Media and Buffers

L-broth. Difco Bacto Tryptone, 10 g/l; Difco Bacto yeast extract, 5 g/l; NaCl, 5 g/l; pH adjusted to 7.2.

L-agar. This is L-broth plus Difco agar, 15 g/l.

R-broth. Sodium pyruvate, 1.5 g/l; sodium hydrogen malate, 1.5 g/l; NH_4Cl , 0.5 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g/l; $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.05 g/l; NaCl, 0.4 g/l; yeast extract, 0.025 g/l; pH adjusted to 6.8 with KOH before autoclaving and 50 ml/l, 0.1 M, potassium phosphate buffer, pH 6.8, added after autoclaving.

R-agar. This is R-broth plus Difco agar, 15 g/l.

BBL-agar. Baltimore Biological Laboratories trypticase, 10 g/l; NaCl, 5 g/l; Difco agar, 10 g/l.

BBL-top layer agar. This is BBL-agar but with only 6.5 g/l of Difco agar.

BBL-top-agarose and L-agarose are identical to BBL-top-agar and L-agar respectively except that agarose, which is used at the same concentration as agar, replaces the latter in each case.

Phage buffer. KH_2PO_4 , 3 g/l; Na_2HPO_4 , 7 g/l; NaCl, 5 g/l; 10 ml/l, 0.1 M MgSO_4 ; 10 ml/l 10 mM CaCl_2 ; 1 ml/l 1% gelatin solution.

Bacterial buffer. KH_2PO_4 , 3 g/l; Na_2HPO_4 , 7 g/l; NaCl, 4 g/l; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g/l.

Antibiotics were used at the following concentrations and added to the molten medium immediately before pouring.

Tetracycline (Tc), 25-50 $\mu\text{g/ml}$; Ampicillin (Amp), 50-100 $\mu\text{g/ml}$; Chloramphenicol base (Cm), 100 $\mu\text{g/ml}$.

Table 2.1 Bacterial strains

	<u>Strain</u>	<u>Genotype</u>	<u>Reference/Source</u>
<u>Rps.capsulata</u>	SP108	<u>pen</u> ^r	Weaver <u>et al</u> (1975)
<u>Rps.capsulata</u>	SP109		J.D. Wall
<u>Rps.capsulata</u>	St. Louis		Weaver <u>et al</u> (1975)
<u>E.coli</u> NM259 =	ED8654	<u>supE</u> , <u>supF</u> , <u>hsdR</u> ⁻ <u>M</u> ⁺ <u>S</u> ⁺ , <u>met</u> ⁻ , <u>trpR</u>	N. Murray
<u>E.coli</u>	ED8538	<u>sup</u> ^o , <u>lacZ</u> _{am}	Murray <u>et al</u> (1977)
<u>E.coli</u>	C600	<u>supE</u> , <u>lac</u> , <u>leu</u> , <u>thi</u> , <u>thr</u> , <u>tonA</u>	Appleyard (1954)

Table 2.2 Phage/Plasmid strains

<u>Strain</u>	<u>Genotype/Relevant Features</u>	<u>Reference/Source</u>
λ NM570BV2	sb1 ⁰ , sb(2-3) [∇] , imm21, nin5, red3	Klein and Murray (1979)
λ 781-0	srI(1-3) [∇] , <u>supE</u> , nin5, imm λ	Murray <u>et al</u> (1977)
λ LS10	srI(1-3) [∇] , <u>pen</u> ⁺ -SP108, nin5, imm λ	This work.
λ LS20	sb1 ⁰ , sb(2-3) [∇] , <u>pen</u> ⁺ -SP108, imm21, nin5, red3	This work.
pMB9	Tc ^r	Bolivar <u>et al</u> (1977a)
pED815	Tc ^r , Cm ^r	Johnson and Willetts (1980)
pSS10	Tc ^r , <u>pen</u> ⁺ -SP108	This work.

1 x TE buffer is:	10 mM Tris-HCl
	1.0 mM EDTA
	pH 7.5
1 x TES buffer is:	50 mM Tris-HCl
	5.0 mM EDTA
	50 mM NaCl
	pH 8.0
1 x SSC buffer is:	150 mM NaCl
	15 mM Trisodium citrate
	pH 7.0
1 x E buffer is:	40 mM Tris-HCl
(for agarose gel	20 mM sodium acetate
electrophoresis)	1.0 mM EDTA
	pH 8.2
	(kept as 10x concentrate)
L buffer consists of:	1.0 ml glycerol
(loading buffer)	0.1 ml 10x E buffer
	0.4 ml 0.5% bromophenol blue
	0.5 ml, 0.5 M EDTA
4 x NT buffer is:	210 mM Tris-HCl
	21 mM $MgCl_2$
	20 $\mu g/ml$ bovine serum albumen (BSA)
	pH 7.5
2 x Poll dilution buffer	2 ml, 1 mg/ml BSA or gelatin
consists of:	100 μl , 2 M $(NH_4)_2 SO_4$
	20 μl , 1 M β -mercaptoethanol
	100 μl , 1 M Tris-HCl, pH 7.5

1 x Denhardt's solution is: 0.02% BSA

0.02% Ficoll (MW ~400,000)

0.02% polyvinyl pyrrolidine

in 6x SSC

10x T4 ligase buffer

660 μ l, 1.0 M Tris-HCl, pH 7.2

consists of:

100 μ l, 0.1 M EDTA

100 μ l, 1.0 M $MgCl_2$

100 μ l, 1.0 M dithiothreitol

10 μ l, 0.1 M ATP

General Note: The centrifugation of small volumes was often carried out in a microcentrifuge (Eppendorf Centrifuge 5412). The tubes used, referred to as microfuge tubes throughout this work, were 1.5 ml polypropylene tubes with lid attached (Sarstedt (UK)).

2.2 Growth Conditions

a. E.coli

All strains were grown, with aeration, in flasks containing L-broth, or on L-agar plates, and were incubated at 37°C. BBL-agar plates were normally used in preference to L-agar plates for the growth of phage.

b. Rps.capsulata

All strains were grown in sealed glass bottles, filled to capacity with R-broth in an illuminated (two or more 40-100 W, tungsten filament, light bulbs) transparent H_2O bath at 30°C. R-plates were incubated in a similarly illuminated fashion at 30°C.

2.3 Lambda Techniques

a. Plating cells

These were prepared by spinning down (10,000g, 5 min) a fresh overnight culture of a suitable strain, usually E.coli NM259, and resuspending in an equivalent volume of 10 mM MgSO_4 .

b. Phage titrations

Serial dilutions of the phage solution were made and 0.1 ml portions of suitable dilutions mixed with 0.2 ml samples of plating cells. These were then added to 2.0 ml aliquots of molten (46°C) BBL-top-agar, mixed, and poured onto BBL plates. Plaques were counted after overnight incubation at 37°C .

c. Plate lysates

A single plaque was transferred into 1 ml phage buffer containing 1 drop of chloroform. Approximately 10^6 plaque forming units (pfu) were then mixed with 0.2 ml of fresh plating cells and 2.5 ml of molten (46°C) BBL-top-agar added. The mixture was then poured onto a freshly prepared L plate and incubated at 37°C until confluent lysis had occurred, (5-8 hr). L-broth (5 ml) (4°C) was then added to the surface of the plate. After overnight incubation at 4°C the broth was removed from the plate and, following addition of one drop of chloroform, shaken gently, clarified (8,000g, 10 min, 20°C) and titrated. Titres were in the range 5×10^9 to 1×10^{11} pfu/ml.

d. Liquid lysates

A fresh overnight culture was diluted 1:50 into L-broth containing: 10 mM MgSO_4 ; glucose, 2% (w/v), and incubated with vigorous shaking at 37°C until the absorbance (650 nm) was equivalent to 0.45 (2×10^8 cells/ml). Phage were then added at a multiplicity of infection (moi) of 1-2 and the culture incubated as before. The turbidity of the culture was measured at 15 min intervals. When this was found to reach a minimum chloroform was added (2 ml/l) and shaking continued for a further 10 min.

e. Precipitation of phage with polyethylene glycol (PEG)

NaCl was dissolved in the culture lysate (40 g/l), followed by RNAase (Sigma) and DNAase I (Sigma) to give concentrations of 1 $\mu\text{g/ml}$ of each. The solution was then left at 20°C for 1-3 hr, with occasional shaking, before being clarified by centrifugation at 10,000 g for 10 min. PEG 6000 was then added, to a final concentration of 10% (w/v), the solution briefly mixed and left overnight at 4°C . The phage were then pelleted (10,000 g, 10 min) and resuspended, in 0.02-0.05 of the original volume of phage buffer, by gentle rotary shaking in the cold room. Further phage purification was by CsCl step gradients and equilibrium gradient centrifugation.

f. Phage preparation by ultracentrifugation

i) CsCl step gradients. The phage suspension was layered onto a preformed CsCl step gradient composed of the following steps: 0.5 ml, saturated CsCl; 0.5 ml, 66% saturated CsCl; 1.0 ml, 50% saturated CsCl; and

1.5 ml, 33% saturated CsCl. The CsCl solutions were made up in phage buffer. Gradients were centrifuged in a 6 x 14 Ti swing-out rotor for 2.5 hr at 120,000 g, 20°C. The phage band was collected from the side of the tube using a syringe and hypodermic needle.

ii) Equilibrium centrifugation. The phage band from the step gradient was mixed with 41.5% pre-clarified CsCl in phage buffer. Centrifugation was in a 6 x 5 Ti swing-out rotor for 24-36 hr at 110,000g, 4°C. The phage band was collected as from the CsCl step gradients.

g. Phage DNA preparations

CsCl was removed from the phage solution by dialysis against TE buffer for 1 hr at 4°C. The suspension was then transferred to a microfuge tube and extracted four times by gentle mixing with an equal volume of freshly distilled phenol (equilibrated with 0.5 M Tris-HCl, pH 7.5). The extractions were carried out on ice. After removal of the final lower phenol layer residual phenol was removed by dialysis against TE buffer at 4°C, with several changes of buffer over a 24 hr period.

h. Preparation of phage DNA from plate lysates (Cameron et al, 1977).

Phage, 10^6 - 10^7 pfu, derived from a single plaque and 0.2 ml of fresh plating cells were added to 2.5 ml of molten (46°C) BBL-top-agarose. This mixture was then poured onto a thick L-agarose plate. The plate was incubated at 37°C until confluent lysis had occurred (5-8 hr), at which time the agar was overlayed with 5 ml

Tris/EDTA solution (10 mM Tris-HCl, pH 7.5; 10 mM EDTA) and left overnight at 4°C. The overlay solution was then poured off and to this was added: 0.4 ml, 0.5 M EDTA; 0.2 ml, 2 M Tris base; 0.2 ml, 10% (w/v) sodium dodecyl sulphate (SDS) (Sigma); and 10 µl diethylpyrocarbonate (DEP) (Sigma). This solution was then heated at 65°C in an open topped tube for 30 min and subsequently cooled on ice. 1 ml of 5 M potassium acetate was added and the solution left for a further 1 hr on ice. Centrifugation, 25,000 g, 10 min was followed by the addition to the supernatant, in a 30 ml Corex (aluminosilicate glass) tube, of 11 ml ethanol. The solution was mixed well and placed in a solid CO₂/ethanol bath at -70°C for 15 min. The solution was allowed to thaw for 5 min at room temperature and the nucleic acids then pelleted by centrifugation at 12,000 g for 1 hr (0°C). The ethanol was then poured off, the tube dried in vacuo and the nucleic acid pellet re-dissolved in 200 µl TE buffer. A 20 µl sample of this solution was found to be adequate for one gel track.

2.4 Plasmid Techniques

a. Preparation of plasmids from E.coli

The following method is based on that of Humphreys et al (1975).

The cells from 1 l of an overnight culture were harvested (10,000 g, 10 min) and resuspended in 10 ml, 25% sucrose (w/v) in 50 mM Tris-HCl, pH 8.0. 1.5 ml lysozyme solution (20 mg/ml in 0.25 M EDTA, pH 8.0) were added and

the mixture swirled intermittently on ice for 5 min. This was followed by the addition of 10 ml, 0.25 M EDTA, pH 8.0, to the solution which was kept on ice for a further 5 min (with occasional mixing) before mixing with 15 ml, Brij/DOC solution (1% Brij 58 (20 cetyl ether) (Sigma), 0.4% (w/v) sodium deoxycholate (DOC) (Sigma) in 10 mM Tris-HCl, 1 mM EDTA, pH 8.0). The Brij/DOC solution was added rapidly from a 25 ml pipette, in order to ensure even mixing, before being left on ice for 20-30 min for lysis to occur.

Cell debris was pelleted by centrifugation (25,000 g, 30 min) and the clear supernatant decanted into a chilled 50 ml measuring cylinder. NaCl, 3% (w/v) and PEG 6000, 10% (w/v) were added, dissolved by inversion, and the solution left to stand overnight at 4°C. The PEG-DNA pellet was precipitated by mild centrifugation (2,000 g, 5 min), the supernatant carefully decanted and the pellet dissolved in 5 ml of TES buffer. CsCl (8 gm) was dissolved in the solution which was then placed on ice for 15-30 min. Under these conditions PEG was displaced from solution and following centrifugation (10,000 g, 10 min) was removed from the top of the tube with a spatula. The supernatant was transferred to a clean tube and 0.5 ml, 10 mg/ml ethidium bromide (EtBr) (Sigma) added. The density of the solution, determined by the weighing of 5 ml samples, was adjusted to 1.59-1.61 gm/ml by the adding of CsCl or TES buffer.

Centrifugation (85,000 g, 40 hr) of the solution in quick-seal tubes (Beckman) produced two DNA bands which

were clearly visible by long wave U/V irradiation. The top band comprised chromosomal and relaxed plasmid DNA and the lower one covalently closed plasmid DNA. The covalently closed plasmid band was collected through the side of the tube with a syringe and hypodermic needle, and the solution extracted three times with an equal volume of n-butanol (equilibrated against a solution of saturated CsCl), in order to remove the EtBr. The plasmid solution was finally dialysed against several changes of TE buffer, at 4°C, over a 24 hr period.

DNA concentration and protein contamination of the solution were estimated by measuring the absorbance at 260 and 280 nm of a ten fold dilution, in TE buffer. Protein contamination was considered to be negligible if the 260/280 ratio was ≥ 1.7 . An absorbance (260 nm) of 1.0 is equivalent to a DNA concentration of about 50 $\mu\text{g/ml}$.

b. Small-scale preparation of plasmids from *E.coli* (for screening purposes)

A 0.5 ml sample of a stationary-phase culture was pelleted in a microfuge (5 min). The pellet was resuspended in 100 μl lysis solution (lysozyme, 2 mg/ml; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA; 50 mM glucose) and left on ice for 30 min before the addition of 200 μl of alkaline SDS solution (0.2 M NaOH; 1% (w/v) SDS) and a further 5 min on ice. 150 μl of a high salt solution (3 M sodium acetate, pH 4.8) were then added, the solution gently mixed and returned to the ice for 1 hr. The sample was then centrifuged for 5 min in a microfuge

and 400 μ l of supernatant removed. After addition of two volumes of ethanol to the supernatant it was placed in a solid CO₂/ethanol bath for 10 min, allowed to thaw, and then re-centrifuged for 5 min in the microfuge. The pellet was re-dissolved in 100 μ l of 0.1 M sodium acetate, pH 6.0, ethanol precipitated as before, and finally dissolved in 100 μ l TE buffer or an appropriate restriction enzyme buffer. A 25 μ l sample of this solution was found to be adequate for a single agarose gel track.

c. Preparation of plasmids from *Rps.capsulata*

Cells from a 1 l early stationary-phase culture were pelleted (10,000 g, 10 min), resuspended (in a 250 ml siliconised flask) in 40 ml of lysis mix (2 mg/ml lysozyme; 25 mM Tris-HCl, pH 8.0; 10 mM EDTA; 50 mM glucose) and, after mixing well, placed on ice for 10 min. 50 ml of alkaline detergent solution (0.2 M NaOH; 1% (w/v) sodium lauroyl sarcosine (Sigma)) were then added and the solution gently mixed for 1 min (until pH = 12.0-12.5). 15 ml of saturated Tris-HCl, pH 7.0, were then immediately added followed by further thorough but gentle mixing for 1 min in order to lower the pH to 8.0-8.5. If a pH value in this range (or lower) was not achieved more saturated Tris-HCl, pH 7.0, was added. Sodium acetate (45 ml, 3 M, pH 4.8) was then added, the lysate mixed and left on ice for 1 hr before being centrifuged (25,000 g, 30 min). The viscous supernatant was decanted and to this was added EtBr (0.5 ml of a 10 mg/ml solution per 20 ml supernatant) and solid CsCl, (to produce a density of

1.58-1.60 gm/ml). The sample was centrifuged (80,000 g, 40 hr) in quick-seal tubes (Beckman) and the plasmid band collected, extracted and dialysed as described above (Section 2.4a).

d. Electron microscopy and size determination of intact plasmid molecules

DNA was spread according to the method of Davis et al (1971). The spreading solution contained: 0.2 µg/ml DNA; 0.1 mg/ml cytochrome C; 0.1 M Tris-HCl, pH 8.5, and 10 mM EDTA, in 50% formamide. A clean glass slide was put into a Petri dish containing hypophase solution (10 mM Tris-HCl, pH 8.5; and 1 mM EDTA, in 15% formamide), 50 µl of the spreading solution slowly applied to the slide/solution boundary and the film thus produced allowed to stand for 1 min before being picked up on Cu grids. The grids were then stained in a freshly prepared solution of uranyl acetate (5×10^{-5} M in 90% ethanol) for 30 s, rinsed in 90% ethanol (10 s), dried on filter paper and shadowed with platinum in a Balzers Evaporator (high vacuum coating unit, Mikro BA 3). The grids were then examined under the Siemens Elmiskop 101 electron microscope, (magnification 20,000x).

Grids containing molecules that it was necessary to photograph were removed from the electron microscope, shadowed with carbon in the evaporator and then returned to the electron microscope for photography using Ilford EM film. After development of the film x5 traces of the negatives were made and these traces measured with a Ferranti Cetec Tablet and Digitiser.

In order to determine the size of plasmid molecules which had been photographed, a standard molecule of known size was included in the appropriate spreadings. The digitised values of several of these molecules, which were present in the same area of the grid square as the molecule whose size was to be determined, used to convert the digitised lengths of molecules to kb. ØX174 (Sanger et al, 1977), taken as being 5.38 kb in size, was the standard molecule used in all such measurements.

2.5 DNA Techniques

a. Ethanol precipitations

DNA was precipitated in siliconised Corex (alumino-silicate glass) tubes or siliconised microfuge tubes. Sodium acetate, 0.1 volumes, 3.0 M, pH 5.5, were added to the solution of DNA followed by mixing and addition of 2-3 volumes of cold ethanol. The solution was then mixed for a second time before being placed in a solid CO₂/ethanol bath for at least 10 min. After permitting the solution to thaw the DNA was pelleted by centrifugation at 12,000 g for 30 min, or 5 min in a microfuge. The supernatant was carefully decanted and the pellet washed by addition of 1-5 ml of cold ethanol (without mixing) and centrifugation at 12,000 g for 5 min or 1 min in a microfuge. The pellet of DNA was dried under vacuum before being re-dissolved in TE buffer.

b. Restriction endonuclease digestions

These were carried out in 1.5 ml microfuge tubes.

Table 2.3 Composition of restriction enzyme buffers (x1).

<u>Restriction Endonuclease</u>	<u>Tris-HCl</u>		<u>NaCl</u>	<u>MgCl₂</u>	<u>β-Mercaptoethanol</u>	<u>Gelatin</u>
	mM	pH	mM	mM	mM	μg/ml
<u>EcoRI</u>	10	7.5	100	10	10	50
<u>HindIII</u>	10	7.5	50	10	10	50
<u>BamHI</u>	10	7.5	50	10	-	50
<u>SalI</u>	10	7.8	150	6	6	50
<u>BglII</u>	10	7.4	60	10	10	50
<u>PstI</u>	6	7.4	50	6	6	50

Appropriate quantities of DNA and restriction endonuclease were incubated with appropriate volumes of distilled H₂O and 10x restriction buffer at 37°C for 1 hr (the total volume was usually 25-40 µl). Digestion was stopped by heating at 70°C for 10 min. Details of the various buffers required by the restriction endonucleases used are given in Table 2.3.

c. Recombination of DNA fragments *in vitro* by the action of T4 ligase.

The restricted DNA was diluted to a concentration of 5-30 µg/ml with 10 mM Tris-HCl, pH 7.5; 0.1 M NaCl. 10% of the final volume of 10x T4 ligase buffer was then added, together with the appropriate quantity of T4 ligase, (the final volume was usually 50 µl). The ligation mixture was incubated for 4-6 hr at 10°C and thereafter (when necessary) stored at -8°C until required for transfection or transformation.

d. Transfection

A fresh overnight culture of the transfection strain E.coli NM259 was diluted (1:50) into L-broth and incubated, with shaking, at 37°C until the absorbance (650 nm) of the culture was 0.55-0.65. The cells were then pelleted (5,000 g, 5 min) in the cold room, and immediately resuspended in ½ initial volume of ice cold 0.1 M MgCl₂. The cells were re-pelleted (as above) and then resuspended in 1/20 initial volume of ice cold 0.1 M CaCl₂, before being placed on ice for at least 30 min. The cells were then regarded as being competent.

DNA [redacted] was diluted into 0.1 ml SSC:CaCl₂ (1 x SSC; 0.1 M CaCl₂, ratio 3:4) and added to 0.2 ml of competent cells. The amount of DNA added did not exceed 0.1 µg. This mixture was gently agitated and placed on ice for 30 min before being subjected to a heat shock, 42°C for 2 min, and returned to the ice for a further 30 min. The cells were then plated out in BBL-top agar in the usual manner.

e. Transformation [redacted]

The procedure was the same as that described above for [redacted] transfection [redacted], except that, after the heat shock the DNA/recipient mixture (0.3 ml) was diluted into 1 ml of L-broth and incubated for 1 hr, at 37°C, before being plated onto appropriate selective media.

f. Horizontal agarose gel electrophoresis of DNA

150 ml of an agarose, 1% (w/v) in 1x E buffer, solution, which had been refluxed for several minutes, was permitted to cool, to approximately 50°C, before being poured into a Perspex (polymethylmethacrylate) mould, 13 cm x 23 cm. The mould contained a Perspex comb, 4 cm from one end, which had twelve teeth. The gel was then left to set at room temperature before the comb was removed and the wells filled with E buffer.

The gel plate was then placed between two buffer tanks, each containing 400 ml of E buffer, and two pieces of Whatman 3 MM chromatography paper, each of which were partially immersed in E buffer, were positioned along the two short edges of the gel, thus creating electrical contact between the gel and the buffer tanks.

Aliquots (5 μ l) of loading buffer (L buffer) were then mixed with each of the DNA samples (usually 25 μ l) to be run. The samples, being more dense than the E buffer, were loaded (with a microsyringe) by underlaying and thus displacing the latter from the wells, which therefore remained full at all times. The surface of the gel was then covered with a transparent, moisture-proof sheet and finally a voltage of 1.5-2.0 v/cm, resulting in a current of 25 amps, applied across the gel. Gels were run for 16-20 hr.

After running the gel was stained by immersion in a solution of ethidium bromide (0.5 μ g/ml) for 30 min and then destained by immersion in H_2O for a similar period of time. DNA bands were visualised by long wave U/V irradiation (Ultra Violet Products Inc) and were photographed, using Ilford FP4 film, through a red filter (Hoya R (25 A)). The exposure time was approximately 5 min.

g. Labelling of DNA with $[^{32}P]$ by nick translation
(Rigby et al, 1977).

A 20 μ l reaction contains the following:

- i) 400 pmoles of each of the three "cold" nucleoside triphosphates. (Final conc. of dNTP's = 20 μ M).
- ii) A sufficient amount of the $[\alpha\text{-}^{32}P]$ -dCTP stock (PB165 from Amersham).
- iii) ≤ 1 μ g DNA to be labelled.
- iv) 25% final reaction volume of 4x NT buffer.

v) 1 μ l DNAase I (Sigma) stock.

(DNAaseI stock = 2×10^{-5} mg/ml DNAaseI, in PolI dilution buffer, 50% glycerol).

vi) 0.5 μ l DNA Polymerase I, 1 unit/ml. (Kindly supplied by Mrs Barbara Will.)

vii) Distilled H_2O \rightarrow final volume (if necessary).

Unlabelled nucleoside triphosphates, DNA, 4 x NT buffer, distilled H_2O , DNAase I and DNA PolI were added to the labelled nucleoside triphosphates. Incubation was at $15^\circ C$, for 1-3 hr, after which time the reaction was stopped, by the addition of EDTA (final conc. 10 mM), and the reaction mixture placed on ice.

Unincorporated labelled nucleotides were then separated from the DNA by passage through a Sephadex G-75 (fine) column, 15 cm x 1 cm diameter, which was eluted with TE buffer. 10 μ l samples of the 1 ml fractions which were collected from the column were counted and those corresponding to the first peak of radioactive material to be eluted from the column were pooled.

In order to check that labelled nucleotides had been incorporated into the DNA of this pool trichloroacetic acid (TCA) precipitable and "total" counts were assayed. As all label in this fraction should be incorporated into DNA and therefore TCA precipitable, the two assays should give the same result.

h. Transfer of DNA from agarose gels to nitrocellulose filters

This procedure is a modification of that initially described by Southern (1975).

The gel was stained or photographed in the usual manner. The gel, or portion of the gel, containing the species of DNA to be transferred was then immersed in 0.5 M NaOH; 1.5 M NaCl, for 40 min before being immersed for the same period of time in 0.5 M Tris-HCl, pH 7.4; 3 M NaCl. The gel was next placed on a sheet of blotting paper soaked in 20 x SSC and which had both ends resting in a reservoir of 20 x SSC. A piece of nitrocellulose, of equivalent dimensions to the gel, was wet with 2 x SSC and laid on the surface of the gel, care being taken to avoid the trapping of air bubbles. A stack of blotting paper, approximately 5 cm high, was then cut to the dimensions of the gel and nitrocellulose. The first sheet of blotting paper was soaked in 2 x SSC and carefully laid on top of the nitrocellulose, again avoiding air bubbles. This was followed by the stack of dry blotting paper being placed on the wet sheet and finally a glass plate being positioned on top of the stack as a weight. The whole apparatus was then covered in a moisture-proof sheet and left overnight for transfer to occur.

After transfer the nitrocellulose filter was rinsed in 2 x SSC, blotted dry, and heated in a vacuum oven at 80°C for 90 min, <5 mm Hg.

i. Hybridisation of DNA bound to nitrocellulose filters and autoradiography

The nitrocellulose filter with bound DNA (see previous section) was incubated in 5 x Denhardt solution, 6 x SSC, at 65°C for 3-4 hr, in order to reduce non-specific DNA

binding. The filter was then placed in a polyethylene bag (sealed along three sides) and the labelled DNA probe, present in a solution of 5 x Denhardt, 6 x SSC, added to it. The volume of this hybridisation solution was kept to a minimum (usually 1-10 ml). The fourth edge of the polyethylene bag was then heat-sealed (Calor, Easyseal), after expulsion of the maximum amount of air possible, and the whole then placed in a second larger polyethylene bag whose edges were again securely sealed. The bag and contents were incubated, with shaking, at 65°C overnight.

When removed from the bag the filter was washed, firstly in 4 x SSC, 0.5% SDS at 65°C for a few hours and secondly in 4 x SSC at 65°C for 1 hr. The solutions were changed two or three times during the washing period.

The filter was finally dried at 37°C before being set up for autoradiography, with Kodak X Omat H film which had been sensitised by pre-flashing. An intensifying screen (Ilford Fast Tungstate) was used and the autoradiograph stored, during exposure, at -70°C.

j. Isolation of total DNA from *Rps.capsulata*

The following procedure is a modification of that described by Marmur (1961).

4 gm of log-phase cells were lysed by incubation at 37°C, for 2-3 hr, in 20 ml: 10 mM Tris-HCl, pH 8.0; 10 mM NaCl; 10 mM EDTA; 0.5% (w/v) SDS and 50 µg/ml Protease K (Sigma). The lysate was spun at 8,000 g for 10 min and the viscous supernatant extracted three times with an equal volume of phenol (equilibrated with

0.5 M Tris-HCl, pH 8.0) and once with an equal volume of chloroform/isoamylalcohol (24:1, v/v). The aqueous layer was then cooled on ice and two volumes of cold ethanol added. The solution was gently stirred with a glass rod and the precipitate collected by spooling onto the rod. The precipitate was then extracted by shaking gently with 1.5 M NaCl (5 ml) at room temperature for 1 hr. This procedure was repeated three times and the extracts combined before being precipitated with ethanol and spooled onto the rod as before. The precipitate was re-dissolved in 10 ml SSC buffer and treated with RNAase (20 µg/ml) at 37°C for 2-3 hr before being phenol extracted, ethanol precipitated and dissolved in TE buffer (1-5 ml). The DNA solution was then subjected to dialysis against this buffer for 24 hr, 4°C, with several changes of the buffer during this period.

2.6 β-Lactamase Techniques

a. Spectrophotometric assays (Waley, 1974)

These were carried out on a Perkin-Elmer 320 spectrophotometer.

Potassium phosphate buffer, 2.5 ml, 0.1 M, pH 6.8 and 10 µl, 30 mg/ml substrate (usually benzylpenicillin) were placed in the sample cuvette and absorbance at the wavelength appropriate to the substrate (Table 2.4) was monitored for 2-3 min in order to ensure that it was constant. A known volume of the solution to be assayed was then added to the cuvette, the contents mixed and the decrease in absorbance monitored. Table 2.4 gives

Table 2.4 Absorbance changes on hydrolysis of 1 M solutions of β -lactamase substrates.

<u>Substrate</u>	<u>Wavelength (nm)</u>	<u>Absorbance change</u>
Benzylpenicillin	232	940
Ampicillin	235	670
Carbenicillin	235	830
Cephaloridine	260	15,000

details of the molar extinction coefficients of the substrates used.

b. Detection of β -lactamase-producing colonies and plaques
(Sherratt and Collins, 1973)

Starch (0.7%, w/v) was incorporated into the media upon which the colonies or plaques were to be grown. After development of colonies or plaques the plates were flooded with 10 ml iodine solution (0.08 M I_2 ; 0.32 M KI; 1% (w/v) sodium tetraborate) for 30-60 s, or until the plates had become stained dark blue-black. The plates were then washed with 10 ml distilled H_2O , drained, and 5 ml of a 1% (w/v) solution of benzylpenicillin in 0.1 M potassium phosphate buffer, pH 6.8, added. The plates were left to stand at room temperature and observed carefully, for 30 min, for the production of zones of clearing by colonies/plaques against the dark background. Colonies or plaques exhibiting this phenomenon were regarded as being produced by bacteria or phage whose genomes coded for an expressible β -lactamase.

c. Isoelectric focusing of β -lactamases in agarose gels

Agarose-EF (LKB) (0.132 gm) and D-sorbitol (LKB) (1.65 gm) were made up to 15.0 ml with distilled H_2O and refluxed for 5 min. The solution was allowed to cool (to approximately $50^\circ C$) and 1 ml of the appropriate Ampholine solution (LKB) added.

Two strips of polyvinylchloride (PVC) tape (each four layers thick) were then placed on a clean glass plate (12.5 cm x 26 cm). The gel solution was mixed and poured

onto the area of glass between the two strips thus forming a gel, 16 cm x 12.5 cm. Excess moisture was removed from the surface of the gel by laying a sheet of Whatman 3MM filter paper on it for 10 min.

A template was placed on the Multiphor 2117 (LKB) cooling plate (set at 10°C) and the glass plate carrying the gel positioned on top of this (liquid paraffin had previously been applied to both the cooling plate and the template). Two electrofocusing strips were cut and to one of them was applied 1.3 ml, 0.5 M NaOH (cathode solution) and to the other 1.0 ml, 0.5 M acetic acid (anode solution). The strips were placed on the gel, according to the markings on the template, as were the sample application strips onto which the samples (10-40 µl) were pipetted. The Multiphor system was then connected to a constant power supply of 5 W. The sample application pieces were removed after 30 min, and focusing regarded as being complete when readily visible marker proteins could be seen to have focused (usually after 2½ hr). When necessary the pH gradient across the gel was determined by placing a pH surface electrode (Russel pH Ltd) along a sample free strip near to the edge of the gel.

β-Lactamase bands were detected by laying over the gel (for a period of 10 s) Whatman 3MM filter paper soaked in Nitrocefin (87/312) (O'Callaghan et al, 1972) (0.5 mg/ml in 0.1 M potassium phosphate buffer, pH 6.8). The intact substrate molecule is yellow but becomes pink when the β-lactam bond is broken, so that focused bands with

β -lactamase activity appear pink on a yellow background. If necessary the Nitrocefin-soaked filter paper was laid across the gel several times.

Photography of the stained gel was with Kodak ortho film type 3 (code no. 2556) through a Kodak Wratten gelatin filter no. 58, colour green, exposure being for 0.125 s at f 5-6.

CHAPTER 3

3.1 Determination of Minimum Inhibitory Concentrations (MIC) Values for Benzylpenicillin

These experiments were carried out on Rps.capsulata strains SP108, SP109 and St. Louis. Cells from mid-exponential-phase cultures, grown in the absence of benzylpenicillin, were diluted in bacterial buffer and 100 μ l samples (containing 50-100 colony forming units (cfu)) were spread onto R plates containing a range of benzylpenicillin concentrations. After incubation for five days the plates were examined and scored for growth (+) or no growth (-) of bacterial colonies (Table 3.1).

Table 3.1 MIC values of strains SP108, SP109 and St. Louis

Strain	Benzylpenicillin concentration (μ g/ml)											
	0	10	20	40	60	80	100	200	400	600	800	1000
SP108	+	+	+	+	+	+	+	+	+	+	+	-
SP109	+	+	-	-	-	-	-	-	-	-	-	-
St. Louis	+	-	-	-	-	-	-	-	-	-	-	-

+ = growth; - = no growth

The MIC was defined as the lowest concentration of benzylpenicillin which prevented visible growth and as can be seen from Table 3.1 the values for SP108, SP109 and St. Louis are 1000, 20 and 10 μ g/ml respectively.

3.2 Plate Detection of β -Lactamase Activity

This test was carried out on Rps.capsulata strains SP108, SP109 and St. Louis. R plates containing starch were prepared and then spread with 10-50 cfu in 100 μ l

Table 3.2 Inducibility of β -lactamase from strain SP108

	Time (min) after addition of benzylpenicillin									
	0	7	15	30	45	60	75	90	105	120
<u>SP108 -</u>										
β -lactamase activity (μ katal/ml culture)	$1.0 \cdot 10^{-3}$	$1.0 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$1.1 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}$	$1.2 \cdot 10^{-3}$	$1.4 \cdot 10^{-3}$	$1.5 \cdot 10^{-3}$	$1.6 \cdot 10^{-3}$	$1.7 \cdot 10^{-3}$
Absorbance (Klett units)	140.0	141.0	142.0	144.5	147.5	150.3	153.5	156.5	159.5	162.0
<u>SP108 +</u>										
β -lactamase activity (μ katal/ml culture)	$9.0 \cdot 10^{-4}$	$2.8 \cdot 10^{-3}$	$1.4 \cdot 10^{-2}$	$3.3 \cdot 10^{-2}$	$5.2 \cdot 10^{-2}$	$7.4 \cdot 10^{-2}$	$9.1 \cdot 10^{-2}$	$1.3 \cdot 10^{-1}$	$1.6 \cdot 10^{-1}$	$1.8 \cdot 10^{-1}$
Absorbance (Klett units)	140.0	141.0	142.0	144.5	147.5	149.5	152.5	155.8	159.0	162.0
<u>SP109 -</u>										
β -lactamase activity (μ katal/ml culture)	$2.6 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	$2.6 \cdot 10^{-4}$	$3.0 \cdot 10^{-4}$	$2.9 \cdot 10^{-4}$	$3.1 \cdot 10^{-4}$	$3.3 \cdot 10^{-4}$	$3.6 \cdot 10^{-4}$	$3.5 \cdot 10^{-4}$	$3.6 \cdot 10^{-4}$
Absorbance (Klett units)	144.5	145.5	146.5	150.0	151.0	152.5	154.5	157.0	161.0	162.0
<u>SP109 +</u>										
β -lactamase activity (μ katal/ml culture)	$3.3 \cdot 10^{-4}$	$2.9 \cdot 10^{-4}$	$2.9 \cdot 10^{-4}$	$3.4 \cdot 10^{-4}$	$3.2 \cdot 10^{-4}$	$4.0 \cdot 10^{-4}$	$3.8 \cdot 10^{-4}$	$3.6 \cdot 10^{-4}$	$4.2 \cdot 10^{-4}$	$4.0 \cdot 10^{-4}$
Absorbance (Klett units)	144.5	145.5	146.5	150.0	151.0	151.5	152.0	152.5	152.5	152.5

- = cultures not induced.

+ = cultures induced with benzylpenicillin (100 μ g/ml)

bacterial buffer. These plates were incubated until colonies were 1-2 mm in diameter and then stained for β -lactamase activity. As can be seen (Fig. 3.1) both SP108 and SP109 gave positive results for this test indicating that both of these strains produce a β -lactamase. The St. Louis strain however does not appear to do so (Fig. 3.2).

3.3 Induction of β -Lactamase Production in Strains SP108 and SP109

Exponential-phase cultures (100 ml) of each strain were divided into two and to one half of each was added benzylpenicillin to give a final concentration of 100 μ g/ml. The 50 ml cultures were then returned to an illuminated water bath and 1 ml samples withdrawn at various times over a 2 hr period. The absorbances of the cultures were recorded (Klett units) at the times at which samples were withdrawn. The 1 ml samples were quickly placed in 1.5 ml microfuge tubes, cooling in a solid CO₂/ethanol bath, and then stored at -20°C. When required the samples were thawed, sonicated (4 x 20 s, 20 KHz bursts), clarified (microfuge, 5 min) and finally assayed for β -lactamase activity. Results are presented in Table 3.2 and Fig. 3.3.

3.4 Substrate Profile of the β -Lactamase from *Rps.capsulata* SP108 and Crypticity Value of this Strain.

This was determined by the assaying for β -lactamase activity of a clarified cellular sonicate of an

Fig. 3.1 Plate detection of β -lactamase production by
Rps.capsulata strains SP108 and SP109
(Section 3.2, p. 48)

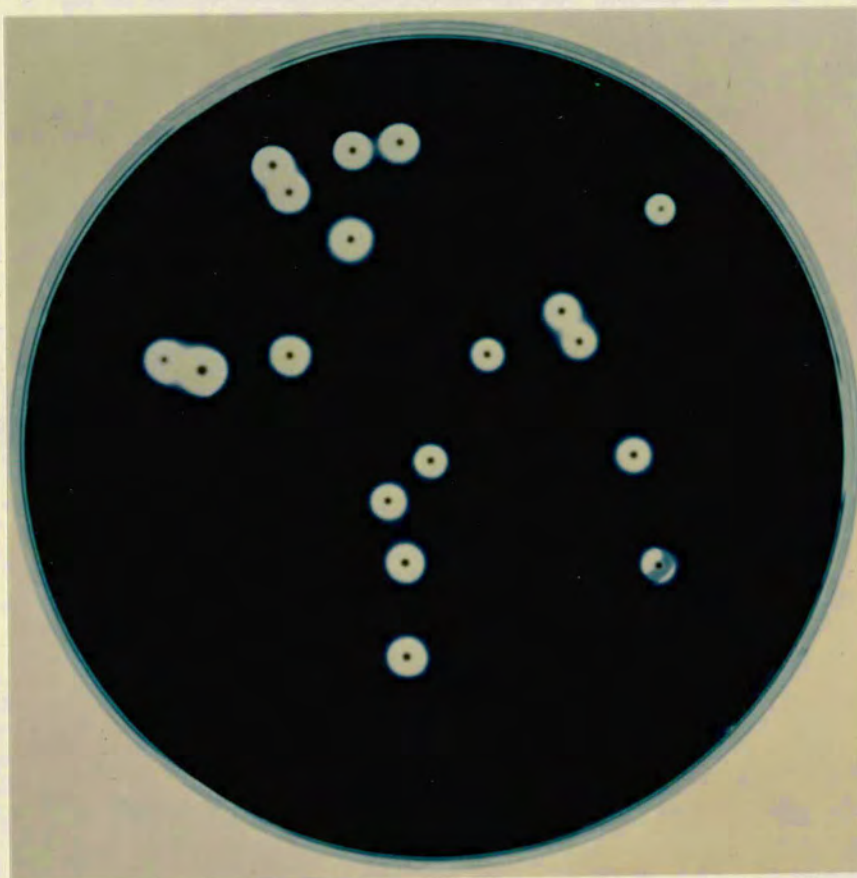
(a) Rps.capsulata strain SP108

(b) Rps.capsulata strain SP109

β -lactamase-producing colonies are surrounded by zones of clearing of the dark background (p. 45).



a.



b.

Fig. 3.1 (a) (b)



Fig. 3.2 Plate detection of β -lactamase production by Rps.capsulata strains SP108, SP109 and St. Louis (Section 3.2, p. 48).

Top left; Rps.capsulata strain SP108
Top right; Rps.capsulata strain SP109
Bottom centre; Rps.capsulata strain St. Louis

β -lactamase-producing colonies are surrounded by zones of clearing, whereas the areas surrounding colonies which do not produce β -lactamase remain dark (p. 45).

Fig. 3.2

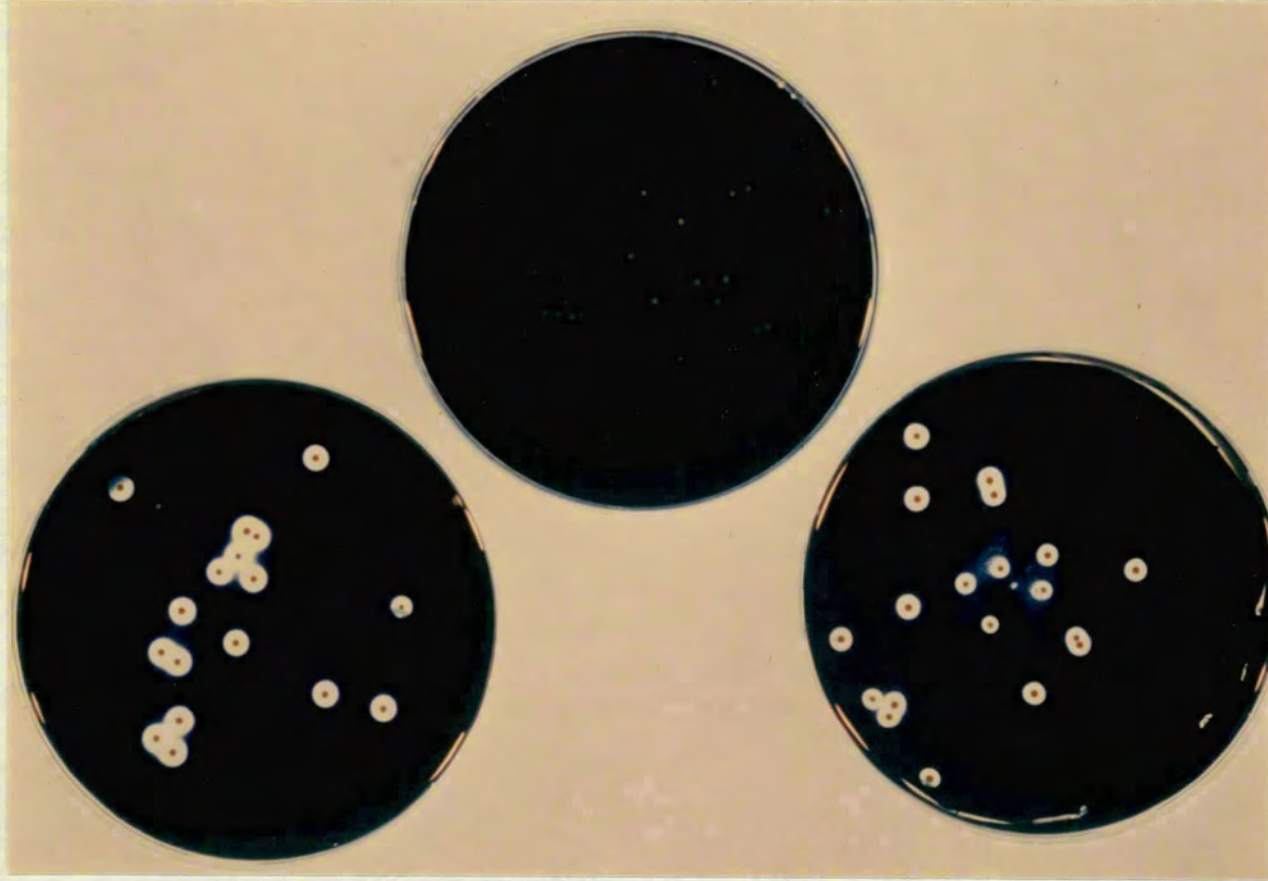


Fig. 3.3 Inducibility of the β -lactamases from Rps.capsulata strains SP108 and SP109 (Section 3.3, p. 49).

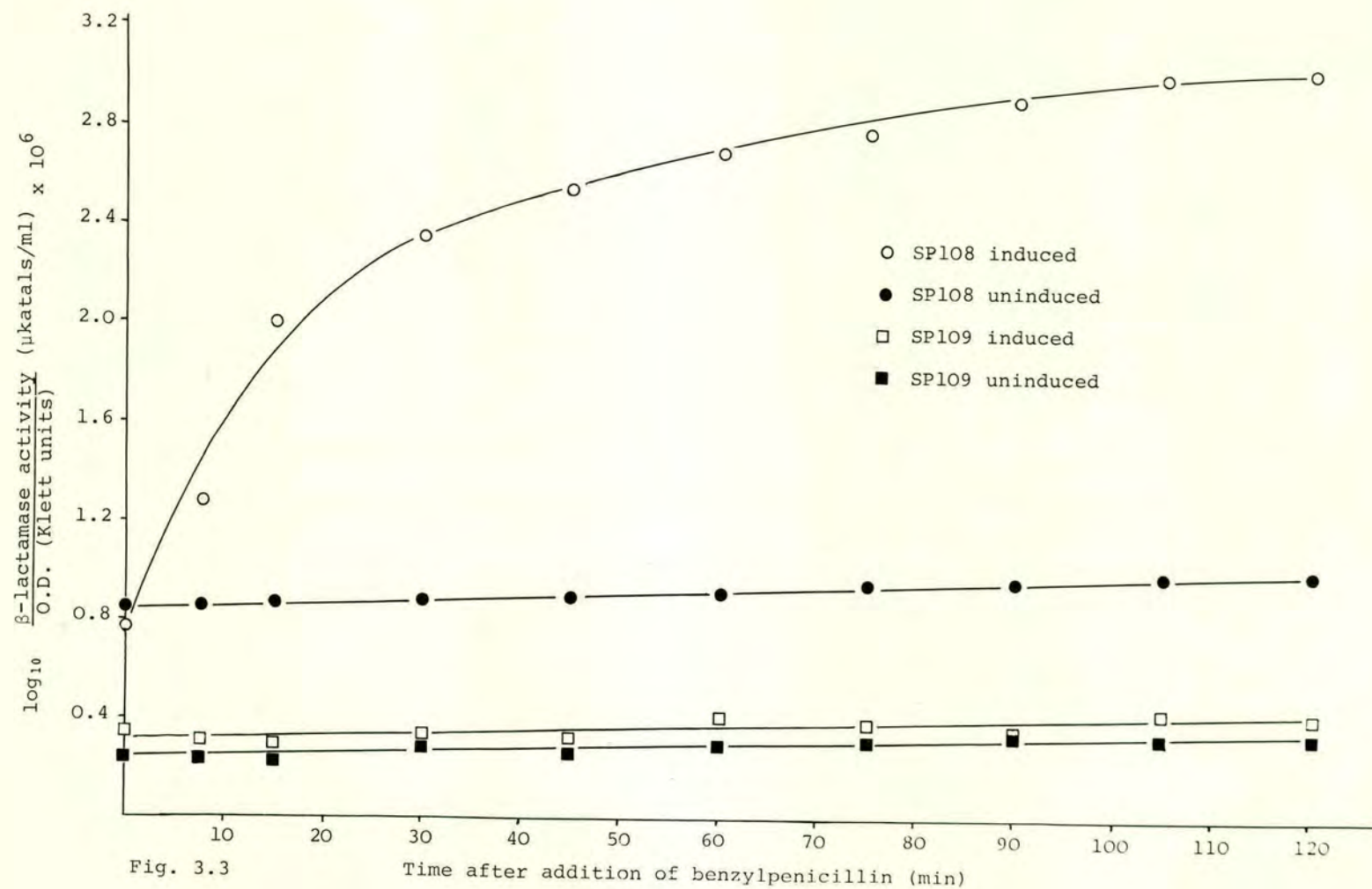


Fig. 3.3

Table 3.3. Substrate profile of the inducible β -lactamase from strain SP108

	<u>Substrate</u>				
	Benzylopenicillin	Ampicillin	Carbenicillin	Cloxacillin	Cephaloridine
Relative rate of hydrolysis	100	27	25	0	4

exponential-phase culture of strain SP108, which had been previously induced with benzylpenicillin. All substrates were used at a concentration of 6 mM, and the profile was calculated relative to an arbitrary value of 100 for the rate of hydrolysis of benzylpenicillin.

The crypicity value (p. 15) of uninduced exponential-phase cells of strain SP108 was found to be 10.

3.5 Cold Osmotic Shock (COS) Treatment of Induced and Uninduced Cultures of *Rps.capsulata* SP108

As stated above (pp. 10-11) this procedure has been used to effect the specific release of certain enzymes from Gram-negative bacteria. The procedure was applied to two late-exponential-phase cultures of strain SP108, one of which had been previously (2 hr) induced with benzylpenicillin and one of which had not. The procedure is described below in some detail.

A 20 ml culture-sample was pelleted in a bench centrifuge (5,000 g, 10 min) and resuspended in 20 ml, 10 mM Tris-HCl, pH 7.3; 30 mM NaCl. The sample was then divided into two and each of the 10 ml samples pelleted as before. One sample was resuspended in 1 ml, 10 mM Tris-HCl, pH 7.3; 30 mM NaCl then placed on ice, this will be referred to as control A. The second sample was resuspended in 1 ml, 20% (w/v) sucrose; 30 mM Tris-HCl, pH 7.3, transferred to a 1.5 ml microfuge tube and EDTA added to a final concentration of 0.1 mM. This solution was then shaken at room temperature for

Table 3.4 Release of β -lactamase from induced (+) and uninduced (-) cells of strain SP108 by cold osmotic shock treatment.

Solution		β -Lactamase activity (μ katal/ml)	
Control A -	(total activity)	7.4×10^{-3}	(100%)
Control B -	(pre-COS-released activity)	7.0×10^{-4}	(10%)
Control C -	(unreleased activity)	2.3×10^{-3}	(31%)
COS supernatant -	(COS-released activity)	4.2×10^{-3}	(57%)
Control A +	(total activity)	8.2×10^{-1}	(100%)
Control B +	(pre-COS-released activity)	3.1×10^{-2}	(4%)
Control C +	(unreleased activity)	2.3×10^{-1}	(28%)
COS supernatant +	(COS-released activity)	4.7×10^{-1}	(57%)

- = solutions derived from uninduced cultures

+ = solutions derived from induced cultures

4 min before the cells were pelleted in the cold room microfuge (5 min). The supernatant was carefully decanted into a second microfuge tube and this (control B) was then placed on ice. The pellet was immediately resuspended in 1 ml, 0.5 mM MgCl_2 (5°C) and placed in a water bath (5°C) where it was shaken for 5 min. This solution was again microfuged (cold room, 5 min) in order to pellet the cells and the cold osmotic shock supernatant (COSS) poured off and stored on ice. The pellet was resuspended in 1 ml, 10 mM Tris-HCl, pH 7.3; 30 mM NaCl, and this solution (control C) also stored on ice.

Cell counts were carried out on samples removed from controls A and C before each of the latter were sonicated (4 x 20 s bursts, 20 KHz output) in order to lyse the cells, and the cell debris pelleted in a cold microfuge (5 min). The lysate supernatants of controls A and C, along with control B and the COSS, were then subjected to β -lactamase assays. The results are presented below in Tables 3.4 and 3.5.

3.6 Isoelectric Focusing of *Rps.capsulata* β -Lactamases

This was carried out on samples control A + and COS Supernatant + from the previous section (Table 3.4), and on the cellular sonicate of a late-exponential-phase culture of *Rps.capsulata* SP109. Before being applied to the isoelectric focusing gel the sonicated sample was clarified (microfuge, 5 min), dialysed overnight against

Table 3.5 Effect of cold osmotic shock treatment on cell viability.

	Viable cell count (cfu/ml)	
	+	-
Control A (before COS)	8.8×10^8	8.2×10^8
Control C (after COS)	6.2×10^8	5.8×10^8
Survival rate	70%	70%

+ = Induced cells

- = Uninduced cells

distilled water at 4°C, lyophilised and resuspended in 1/10th of its original volume of distilled water.

As can be seen (Fig. 3.4) samples from SP108 (control A +) and SP109 when focused produced two bands of activity at positions corresponding to pI values of 4.5 and 4.7. The β -lactamase activity of the cold osmotic shock supernatant however was present mainly at the pI 4.5 position.

3.7 Analyses of *Rps.capsulata* Plasmid DNA.

a. Electron microscopic analysis

Plasmid DNA which had been isolated from *Rps.capsulata* strains SP108, SP109 and St. Louis in the manner described above (Section 2.4c), was subjected to an electron microscopic analysis which distinguished the following classes of plasmids (Table 3.6).

b. Restriction endonuclease analysis

The restriction patterns of plasmid preparations from strains SP108 and SP109, which were generated by digestion with restriction endonuclease EcoRI, are indistinguishable (Fig. 3.5, tracks 1 and 2).

Restriction enzyme analyses were also carried out on plasmids prepared from cultures of strain SP108 grown in the presence of (500 μ g/ml) and absence of benzylpenicillin. Fig. 3.5 (tracks 3-9) shows that no visible changes in the restriction pattern of the plasmids correlate the alternative conditions of growth.

It can be seen (Fig. 3.5) that restriction digests of

Table 3.6. Sizes of plasmids from strains of Rps.capsulata

<u>Strain</u>	<u>Plasmid sizes (kb)</u>			
SP108	i) 127.0 (1.1)	ii) 92.0 (1.6)	iii) 14.9 (0.74)	iv) 8.4 (0.37)
SP109	i) 126.8 (6.7)	ii) 91.7 (2.5)	iii) 15.0 (0.54)	iv) 8.4 (0.25)
St. Louis	i) 77.8 (1.4)			

Standard deviations are in parentheses, and size estimations are the mean of several determinations (minimum number 5).

Fig. 3.4 Isoelectric focusing of β -lactamases from Rps.capsulata (p. 51).

<u>Track number</u>	<u>Sample</u>
1	cytochrome c-551 (marker)
2	<u>Rps.capsulata</u> SP108 total β -lactamase activity
3	<u>Rps.capsulata</u> SP108 COS-released β -lactamase activity
4	azurin (marker)
5	cytochrome c-551 (marker)
6	<u>Rps.capsulata</u> SP109 total β -lactamase activity
7	azurin (marker)

Isoelectric focusing was carried out in an agarose gel and the pH gradient generated by pH 4.0-6.5 Ampholines (LKB) (see p. 45).

Notes:

- (i) Tracks 2 and 3 indicate that cold osmotic shock-released β -lactamase from strain SP108 consists mainly of the pI 4.5 species of activity.
- (ii) Tracks 2 and 6 indicate that the β -lactamases of strains SP108 and SP109 have the same pI values.

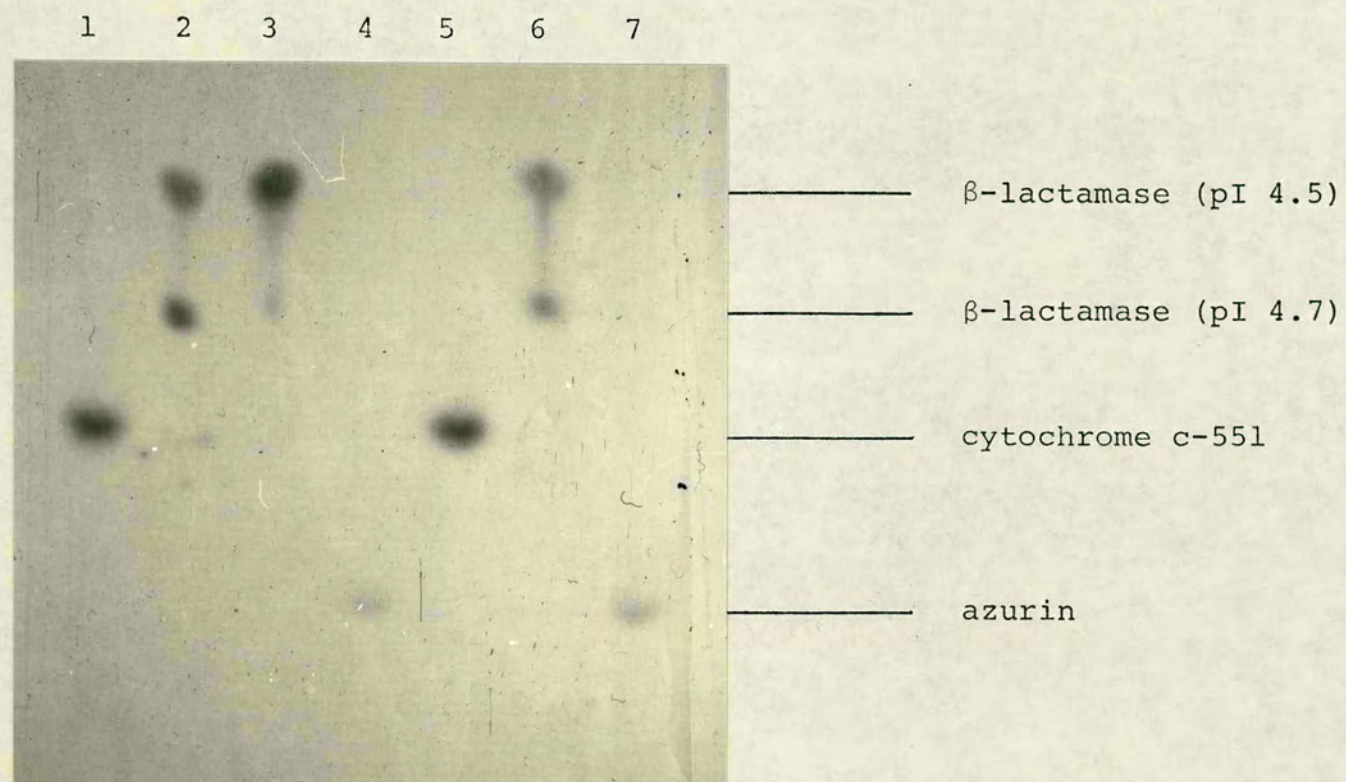


Fig. 3.4

Fig. 3.5 0.7% agarose gel electrophoresis of DNA fragments.

<u>Track number</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	Plasmid preparation from <u>Rps.capsulata</u> SP108 (-)	<u>EcoRI</u>
2	Plasmid preparation from <u>Rps.capsulata</u> SP109 (-)	<u>EcoRI</u>
3	Plasmid preparation from <u>Rps.capsulata</u> SP108 (+)	<u>EcoRI</u>
4	Plasmid preparation from <u>Rps.capsulata</u> SP108 (-)	<u>EcoRI</u>
5	<u>λcI857</u>	<u>HindIII</u>
6	Plasmid preparation from <u>Rps.capsulata</u> SP108 (+)	<u>BamHI</u>
7	Plasmid preparation from <u>Rps.capsulata</u> SP108 (-)	<u>BamHI</u>
8	<u>λcI857</u>	<u>HindIII</u>
9	Plasmid preparation from <u>Rps.capsulata</u> SP108 (+)	<u>HindIII</u>
10	Plasmid preparation from <u>Rps.capsulata</u> SP108 (-)	<u>HindIII</u>

+ = plasmid preparations derived from cultures grown in the presence of benzylpenicillin (500 µg/ml).

- = plasmid preparations derived from cultures grown in the absence of benzylpenicillin.

Notes:

- (i) Fragments in marker track (S) are derived from a HindIII digestion of λcI857 (sizes expressed in kb).
- (ii) There are no observable differences between the restriction patterns of plasmids derived from strains SP108 and SP109 (tracks 1 and 2).
- (iii) There are no observable differences between SP108 plasmid preparations derived from cultures grown in the presence and absence of benzylpenicillin.

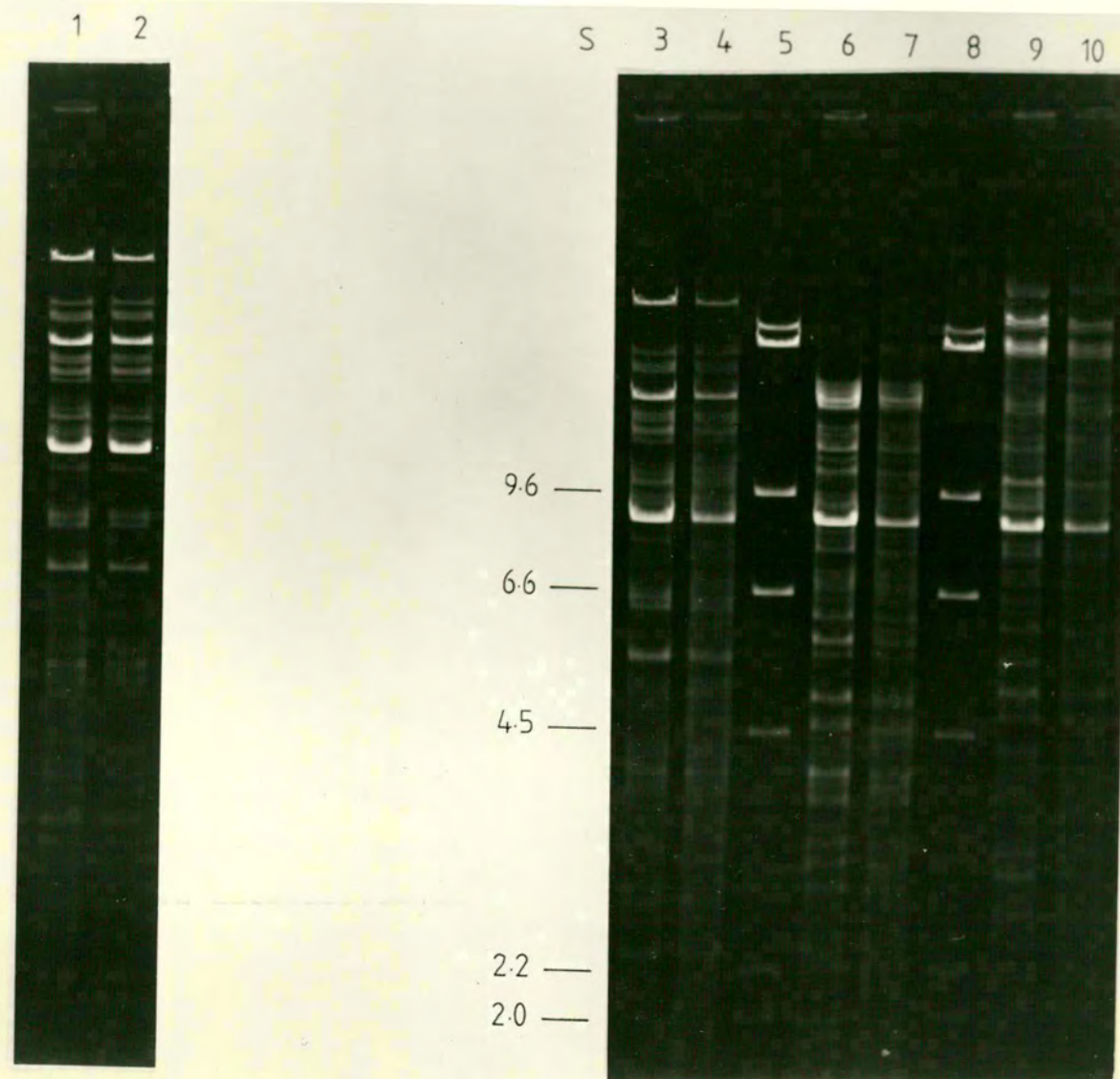


Fig.3.5

any of the plasmid preparations from strains SP108 and SP109 consist of a larger number of bands with relative intensities that are not related to the sizes of the restriction fragments that they represent. This is probably because the four plasmid species of these strains may not be present in the plasmid preparations in equimolar amounts. The most striking example of this varying intensity of bands is illustrated in Fig. 3.6 (tracks 1-3). Here digestion of an SP108 plasmid preparation with each of the restriction endonucleases HindIII, BamHI and EcoRI can be seen to generate a particularly intense band at a position corresponding to an 8.5 kb restriction fragment. This band is of sufficiently high intensity to permit recognition, after simultaneous digestion with two restriction endonucleases, of the sub-fragments of which it is composed. This procedure can be used to produce a restriction enzyme map of the molecular species giving rise to the intense band (Fig. 3.7(a)).

A particular plasmid preparation, derived from strain SP108, was observed to no longer generate the intense 8.5 kb band on digestion with HindIII, BamHI and EcoRI. This plasmid preparation will be referred to as SP108-1. It was further observed (Fig. 3.6, tracks 10-12) that the 8.5 kb band is, in this preparation, replaced by an equally intense band at a position corresponding to that of an 11.2 kb restriction fragment. A restriction map of this larger molecular species was derived, as for the

Fig. 3.6 0.7% agarose gel electrophoresis of DNA fragments.

<u>Track number</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	SP108 plasmid preparation	<u>EcoRI</u>
2	SP108 plasmid preparation	<u>HindIII</u>
3	SP108 plasmid preparation	<u>BamHI</u>
4	<u>λ</u> cI857	<u>HindIII</u>
5	SP108 plasmid preparation	<u>EcoRI</u> / <u>HindIII</u>
6	<u>λ</u> cI857	<u>EcoRI</u> / <u>HindIII</u>
7	SP108 plasmid preparation	<u>EcoRI</u> / <u>HindIII</u>
8	<u>λ</u> cI857	<u>EcoRI</u>
9	SP108 plasmid preparation	<u>HindIII</u> / <u>BamHI</u>
10	SP108-1 plasmid preparation *	<u>EcoRI</u>
11	SP108-1 plasmid preparation	<u>HindIII</u>
12	SP108-1 plasmid preparation	<u>BamHI</u>
13	<u>λ</u> cI857	<u>HindIII</u>
14	SP108-1 plasmid preparation	<u>EcoRI</u> / <u>HindIII</u>
15	<u>λ</u> cI857	<u>EcoRI</u> / <u>HindIII</u>
16	SP108-1 plasmid preparation	<u>EcoRI</u> / <u>BamHI</u>
17	<u>λ</u> cI857	<u>EcoRI</u>
18	SP108-1 plasmid preparation	<u>HindIII</u> / <u>BamHI</u>

* See p. 53 for a description of the SP108-1 plasmid preparation.

Notes:

- (i) Fragments in marker tracks (S) and (T) are derived from HindIII and EcoRI digestions of λcI857 (sizes expressed in kb).
- (ii) The intense bands[†], tracks 1-3 and 10-12, are labelled 8.5 kb and 11.2 kb respectively.
- (iii) Sub-fragments derived from the intense bands are present in tracks 5, 7 and 9, and tracks 14, 16 and 18.

[†] See text (p. 53).

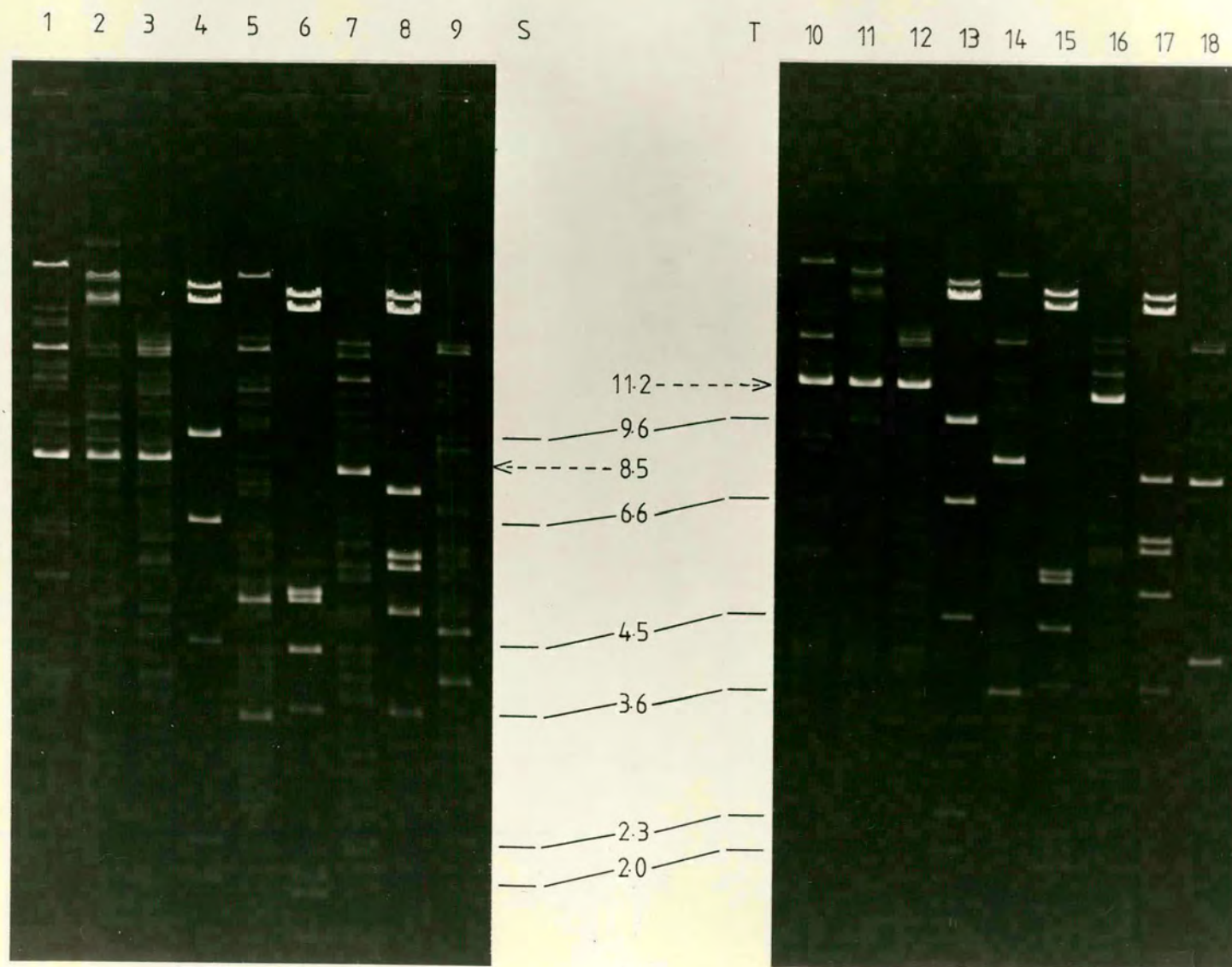


Fig.3-6

Fig. 3.7 Restriction maps (EcoRI, HindIII and BamHI) of plasmids isolated from Rps.capsulata.

(a) 8.5 kb plasmid from an SP108 preparation

(b) 11.2 kb plasmid from an SP108-1 preparation

Notes:

- (i) The maps presented here are based on data derived from Fig. 3.6.
- (ii) Distances between restriction sites are expressed in kb.

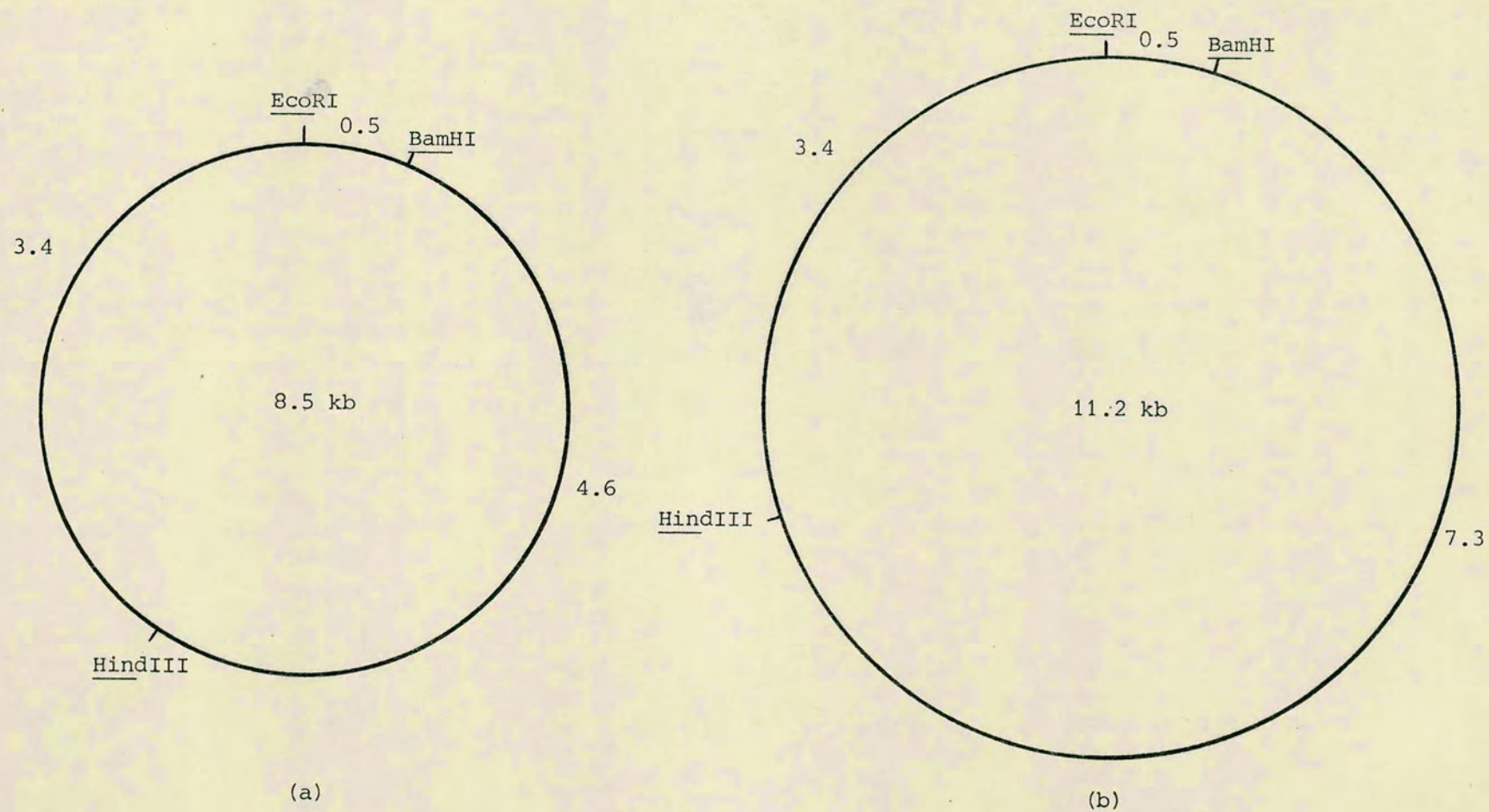


Fig. 3.7

8.5 kb band-forming species of DNA, and is presented in Fig. 3.7 (b).

Fig. 3.7 (a) shows that the molecule giving rise to the 8.5 kb intense band is an 8.5 kb circle of DNA and is therefore probably the 8.4 kb plasmid initially observed under the electron microscope. It can also be seen (Fig. 3.7 (b)) that the molecule producing the 11.2 kb band is circular. It would appear from the restriction patterns of these two plasmids that the larger one has arisen from the acquisition, by the 8.4 kb plasmid, of an extra 2.7 kb of DNA. The extra DNA is present between the unique BamHI and HindIII sites.

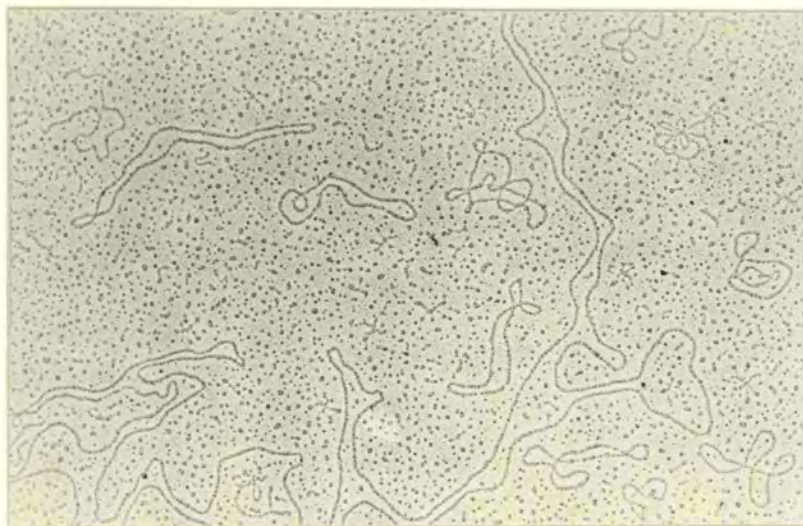
The presence of a fifth class of plasmid in the SP108-1 preparation has been substantiated by an electron microscopic analysis (Fig. 3.8). This indicated the existence, in addition to the plasmids present in strain SP108, of a plasmid whose size (as estimated by contour length measurements) is 11.4 kb (standard deviation 0.24).

Fig. 3.8 Electron micrographs of Rps.capsulata plasmid DNA (magnification 40,000x).

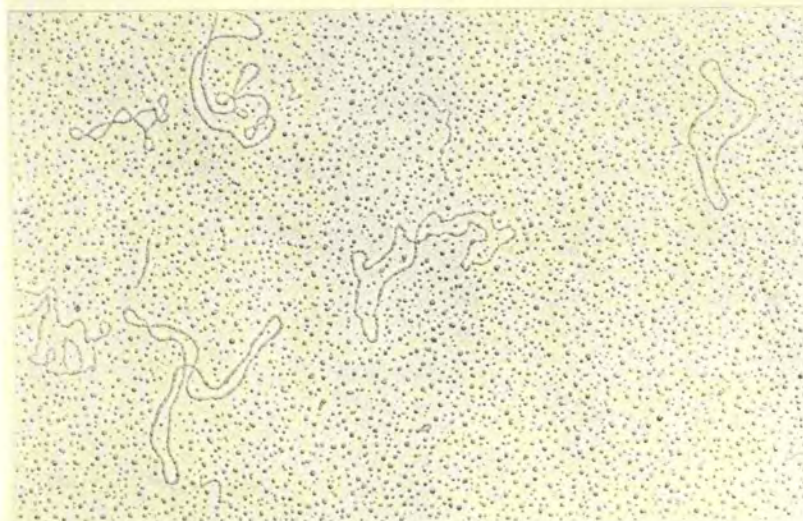
- (a) SP108 plasmid preparation.
- (b) SP108-1 plasmid preparation.

The keys identify the positions of 8.4 kb (key (a)) and 11.4 kb (key (b)) plasmids.

ØX174 (5.4 kb) is present on both electron micrographs as a "standard" molecule.

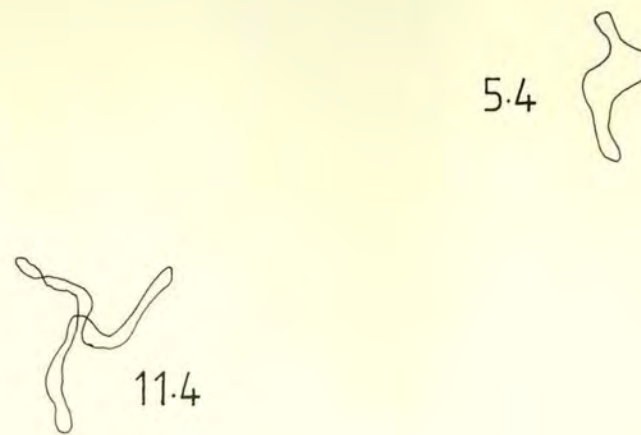
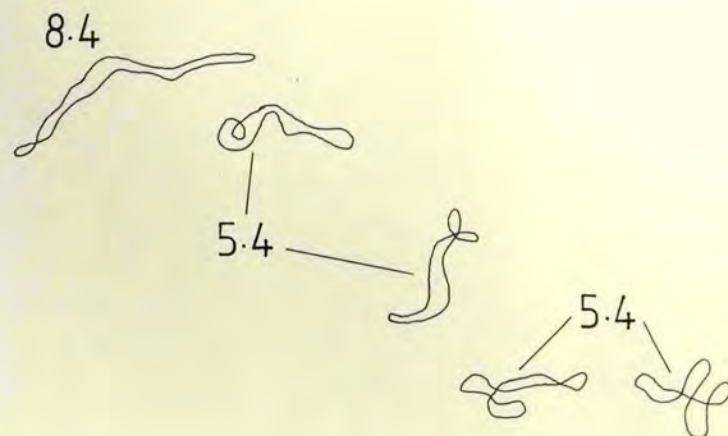


a



b

Fig. 3.8



CHAPTER 4

4.1 Attempted Isolation of Penicillin-Sensitive Derivatives of *Rps.capsulata* SP108

In order to determine whether or not the β -lactamase of this strain was plasmid-mediated attempts were made to isolate colonies which no longer possessed a Pen^r phenotype. The plasmid complements of such strains could then be examined to see if the change in phenotype had been accompanied by the loss of a plasmid.

Samples of an exponential-phase culture of SP108, which was grown in the absence of benzylpenicillin, were diluted in bacterial buffer^{and}/plated onto R plates. The susceptibility of the resulting colonies to penicillin was then examined by transferring them (with toothpicks) onto R plates containing (100 μ g/ml) and not containing benzylpenicillin. Approximately 2000 colonies were examined in this way, none of which proved to be Pen^s.

The curing agent EtBr was then used to try and encourage loss of a plasmid. Strain SP108 was grown in a range of EtBr concentrations in order to select a concentration which significantly inhibited, but did not abolish, growth of the culture. Such a concentration was found to be 5×10^{-6} M, and therefore EtBr, at this level, was incorporated in the growth medium of a culture from which single colonies were isolated.

The colonies were then treated, as described above, in order to detect any Pen^s isolates. Approximately 4000 colonies were examined and none of them found to be penicillin-sensitive.

Although the examination of a total of 6000 colonies is by no means exhaustive in this type of study and does not preclude the possibility that the β -lactamase gene is resident on a disposable plasmid, it was felt that alternative approaches to this problem should be pursued.

4.2 Attempted Isolation of Penicillin-Resistant Derivatives of *Rps.capsulata* SP109

A late-exponential-phase culture of SP109 was diluted into R-broth; R-broth plus benzylpenicillin (50 $\mu\text{g/ml}$); and R-broth plus benzylpenicillin (100 $\mu\text{g/ml}$), to give concentrations of 10^6 cfu/ml. A range of samples containing 10^6 - 10^8 cfu were then taken from each of the three dilutions and incubated in sealed glass containers.

All samples from the R-broth not containing benzylpenicillin showed visible signs of growth after 48 hr. However, all samples from R-broth containing benzylpenicillin (50 and 100 $\mu\text{g/ml}$) did not show any signs of growth after four weeks of incubation.

4.3 Molecular Cloning of the *Rps.capsulata* β -Lactamase using Vector pMB9

In order to be able to determine the nucleotide sequence of the SP108 β -lactamase structural gene it is necessary to produce sufficient quantities of the corresponding DNA sequence in a purified form. This can be achieved by the molecular cloning of the gene.

As it was unknown at this stage whether the structural gene had a plasmid or chromosomal location total DNA

(chromosomal plus plasmid) was prepared from strain SP108 and used as the source of the β -lactamase gene in the initial cloning experiments.

The plasmid vector pMB9 (Bolivar et al, 1977a) confers resistance to the antibiotic tetracycline (Tc) but not to penicillin. It contains single sites for the restriction endonucleases EcoRI, HindIII, BamHI and SalI. The sites for the last three enzymes are all within or close to the region encoding resistance to tetracycline, so that insertion of DNA at any of these sites abolished the Tc^r phenotype. As tetracycline is the only antibiotic to which pMB9 confers resistance insertions of this type prevent an antibiotic-resistance selection of pMB9 transformants. For this reason it was decided to clone into the EcoRI site of pMB9.

An EcoRI-digested sample of total DNA from SP108 was ligated to similarly cleaved pMB9 molecules. Competent cells (E.coli NM259) were transformed with the ligation mixture and attempts made to select, and screen for, E.coli transformants which had acquired the SP108 β -lactamase gene. The selection was carried out by plating transformants onto media containing tetracycline and benzylpenicillin (100 μ g/ml). The screen used for the detection of β -lactamase production was the starch plate method.

Neither of the methods detected any penicillin-resistant transformants.

Recombinant molecules containing the SP108 β -lactamase gene should occur at a frequency of not less than 1 in 600

pMB9 recombinants, as the genome size of Rps.capsulata is likely to be similar to that of Rps.sphaeroides (1.6×10^3 Md) (Gibson and Niederman, 1970), and should therefore produce approximately 600 DNA fragments upon digestion with a hexanucleotide sequence recognition enzyme such as EcoRI. The failure of the selection and screen tests to identify any such recombinants after the examination of 4000 presumed recombinants therefore suggests that either there is an EcoRI site within the β -lactamase structural gene, or that the appropriate recombinant is being formed but not detected. Possible reasons for an inability to detect such a recombinant are that the gene is not being expressed, or its product not normally processed. In view of these difficulties, and in order to differentiate between the two types of possible cause, attempts were made to clone the β -lactamase gene in a phage vector.

4.4 Molecular Cloning of the Rps.capsulata β -Lactamase using Vector λ 781-0

The phage vector λ 781-0 (Murray et al, 1977) is an EcoRI replacement vector and thus has two sites for EcoRI which flank a replaceable segment of the phage genome. The main purpose of this central fragment is to provide the phage with sufficient DNA to prove viable, as a phage with too little DNA is not capable of forming plaques (Bellet et al, 1971). Thus, viable phage emerging from a ligation reaction with EcoRI total DNA from SP108 must

have regained this original fragment or acquired Rps.capsulata DNA in its place. These two possibilities are clearly distinguishable as the original fragment carries a mutant tRNA gene, supE, which confers a readily recognisable phenotype on phage that carry it. The presence of the supE gene can be detected by its ability to suppress an amber mutation in the lacZ gene of the bacterial host E.coli ED8538. This combination of host and phage therefore produce blue plaques on agar containing 5-bromo-3-chloro-2-indolyl- β -D-galactoside (Davies and Jacob, 1968), due to the hydrolysis of this substrate by the lacZ gene product (Smith et al, 1970).

The screen employed for detection of recombinant phage carrying the β -lactamase gene of SP108 is an adaptation of the starch plate test for β -lactamase-producing colonies. This adaptation was first tested with λ 781-O and a derivative, λ 781-BV2 (kindly provided by Binie Klein), which contains the plasmid pBR322 (Bolivar et al, 1977b) carrying a TEM-type β -lactamase, in place of the original supE fragment. Plaques formed by λ 781-O when developed to detect β -lactamase activity (by the starch plate test) remained dark whilst those formed by λ 781-BV2 produced large areas of clearing, indicating that this test works equally well for plaques or colonies.

Transfection of E.coli NM259, with a sample of a ligated mixture of SP108 total and λ 781-O DNA cut with EcoRI, yielded 3000 plaques of which approximately 70% were recombinants. Screening of these plaques for β -lactamase activity detected one plaque which gave a

Fig. 4.1 Plate detection of β -lactamase-producing phage (p. 59).

Left; plaques formed by a β -lactamase-producing phage, λ LS10.

Right; plaques formed by a non- β -lactamase-producing phage, λ NM570BV2.

Plaques formed by phage which carry a β -lactamase gene are surrounded by a zone of clearing.

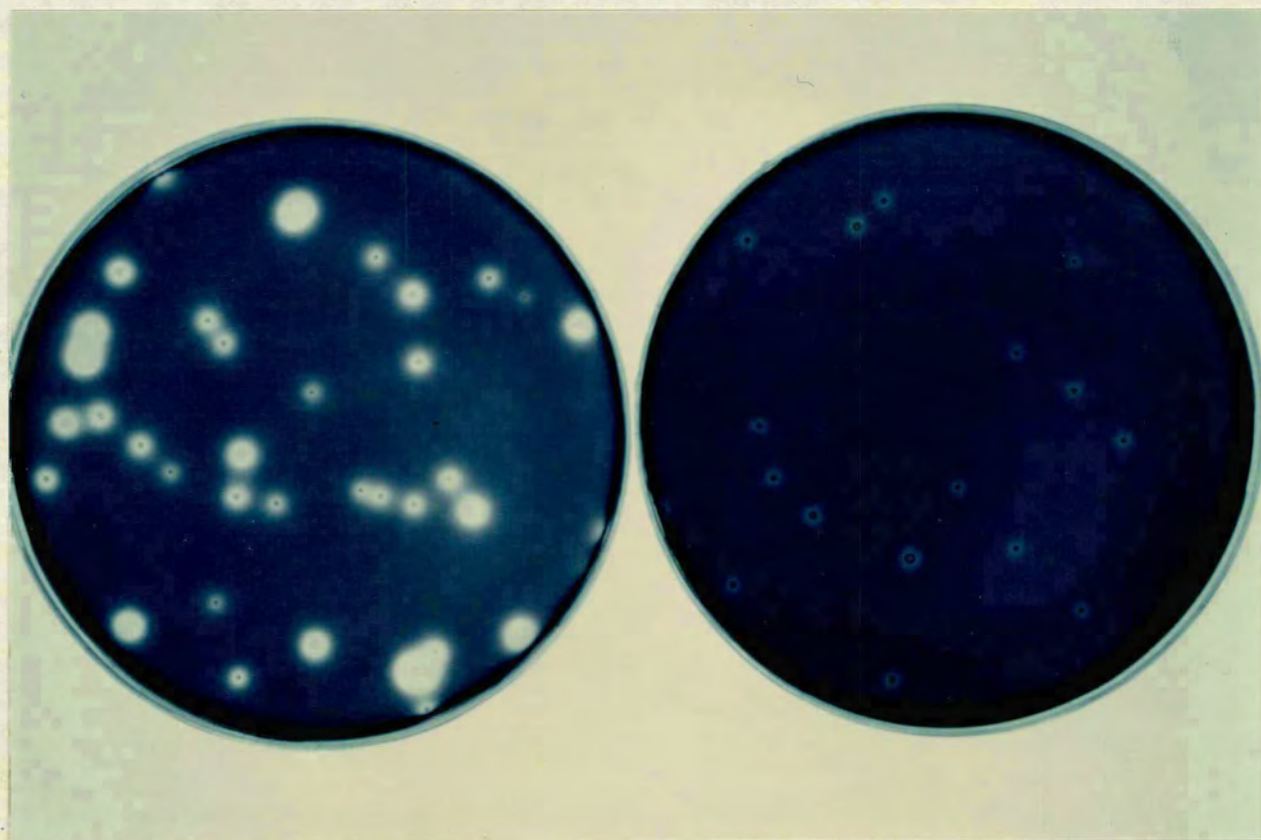


Fig. 4.1

positive result (see Fig. 4.1). This plaque was immediately picked into 1 ml phage buffer and a large scale preparation made of a phage purified from it. This recombinant phage will be referred to as λ LS10. Restriction of λ LS10 DNA with EcoRI (Fig. 4.2(a), track 4) illustrated that a single EcoRI fragment of approximately 12 kb had replaced the supE-carrying central fragment of λ 781-0. It was therefore supposed that this 12 kb fragment carried the β -lactamase of Rps.capsulata SP108. In order to confirm this supposition attempts were made to identify the β -lactamase of λ LS10 using the technique of isoelectric focusing.

The decrease in absorbance (650 nm) of a liquid lysate of λ LS10 was carefully followed until found to be a minimum, whereupon, the lysate was clarified (10,000 g, 10 min), without prior addition of chloroform, and samples of the supernatant were subjected to isoelectric focusing, along with appropriate controls and markers. Isoelectric focusing indicated that λ LS10 encodes for a β -lactamase activity which was represented by two bands, at positions corresponding to pI values of 4.5 and 4.7, and is thus identical to the activity previously detected in Rps.capsulata strains SP108 and SP109. The original vector λ 781-0 was not found to encode any such activity.

With the intentions, firstly of demonstrating that the 12 kb fragment is Rps.capsulata DNA, and secondly of determining the genomic location of this fragment within its native species, α - $[^{32}\text{P}]$ -labelled λ LS10 DNA was hybridised against EcoRI-digested DNA which had been

Fig. 4.2

(a) 0.7% agarose gel electrophoresis of DNA fragments.

<u>Track number</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	Plasmid preparation from <u>Rps.capsulata</u> SP108	<u>EcoRI</u>
2	Plasmid preparation from <u>Rps.capsulata</u> SP109	<u>EcoRI</u>
3	λ 781-0	<u>EcoRI</u>
4	λ LS10	<u>EcoRI</u>
5	pED815	<u>EcoRI</u>
6	pSS10	<u>EcoRI</u>
7	pBR322	<u>EcoRI</u>

(b) Hybridisation analysis of Southern transfers of the DNAs from gel (a) using a ^{32}P -labelled λ LS10 DNA probe.

Notes:

- (i) Fig. 4.2 (b) tracks 1 and 2 indicate that λ LS10 DNA hybridises to 12.4 kb EcoRI fragments present in the plasmid DNA preparations from Rps.capsulata strains SP108 and SP109.
- (ii) λ LS10 DNA can also be seen to hybridise to the 12.4 kb EcoRI insert of pSS10 (track 6).

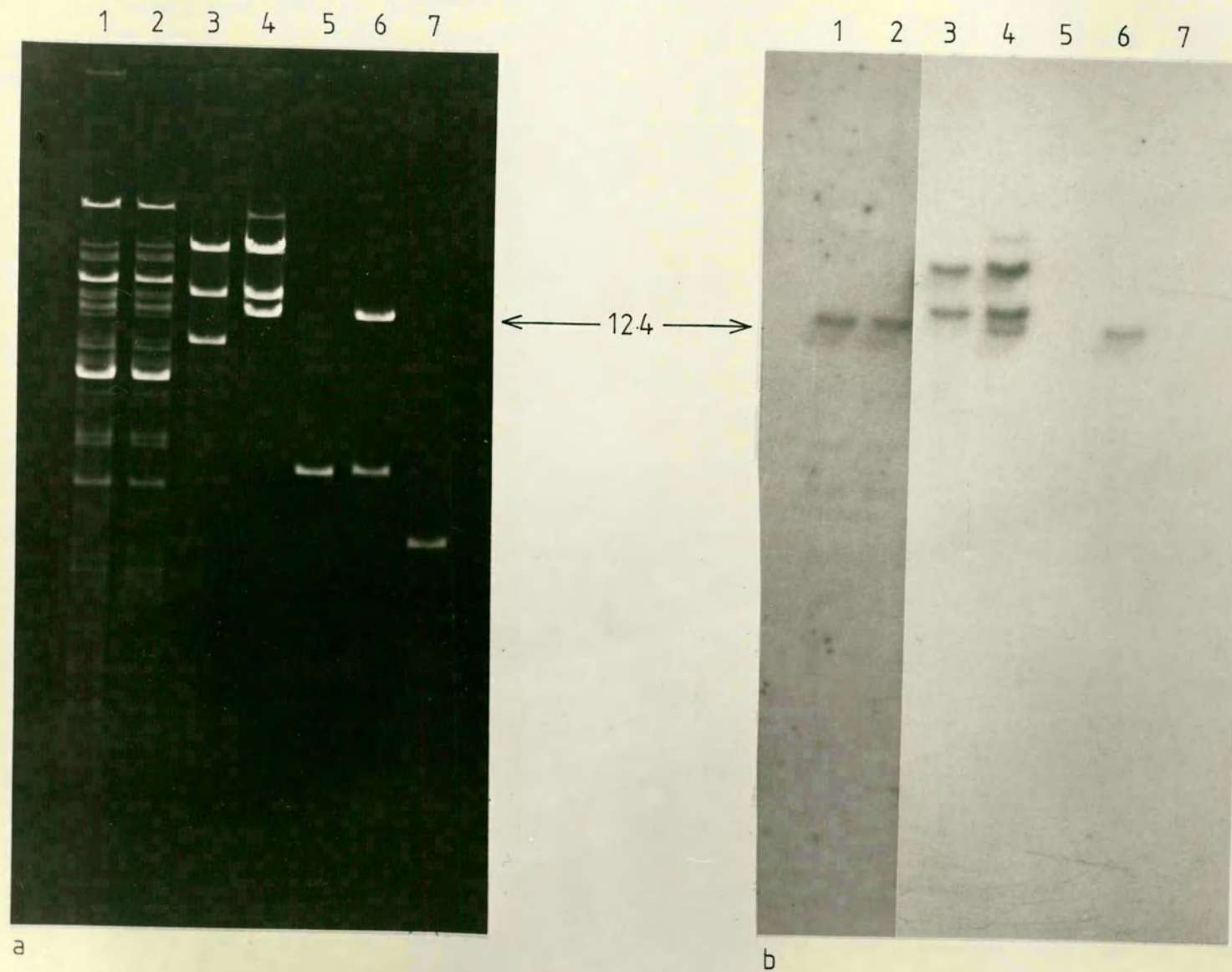


Fig. 4.2

bound to a nitrocellulose filter by the technique of Southern (1975). The samples of EcoRI-digested DNA bound to the filter were: SP108 plasmid DNA; SP109 plasmid DNA; λ 781-O DNA; λ LS10 DNA; and pBR322 DNA. Apart from hybridising strongly to itself and the left and right arms of λ 781-O, λ LS10 also hybridised to an EcoRI fragment present in the plasmid preparations of strains SP108 and SP109. This fragment was the same size as the λ LS10 insert (approximately 12 kb) thus confirming that this phage contains a fragment of Rps.capsulata DNA, and indicating a plasmid location for this fragment in Rps.capsulata strains SP108 and SP109 (see Fig. 4.2 (b)).

4.5 Transfer of the Rps.capsulata β -Lactamase Gene to pED815

The above results confirm that the SP108 β -lactamase has been transferred on a 12 kb EcoRI fragment to vector λ 781-O. This suggests that the reason for failure to detect an analogous transfer to the vector pMB9 was caused by expression or processing problems in the plasmid system. In order to investigate this further the β -lactamase-carrying fragment was transferred from λ LS10 to the plasmid vector pED815. This vector is a pBR325 (Bolivar, 1978) derivative, which has suffered a deletion of approximately 0.5 kb (N.S. Willetts, personal communication) of the amp^r gene, thus rendering the plasmid Amp^s, Tc^r, Cm^r. Vector pED815 contains a unique EcoRI cleavage site in the chloramphenicol resistance gene, and

Table 4.1 β -Lactamase activity of E.coli strains carrying recombinant plasmids.

<u>Strain</u>	<u>β-lactamase activity of cultures</u>	
	(μkatal/ml)	
	+	-
<u>E.coli</u> NM259	nd	nd
<u>E.coli</u> NM259 (pED815)	nd	nd
<u>E.coli</u> NM259 (pSS10)	nd	nd
<u>E.coli</u> NM259 (pBR322)	4.3×10^{-1}	4.5×10^{-1}

nd = none detectable

β -lactamase activities presented were determined by the dilution of overnight cultures 1:20 into L-broth with (+) (100 μg/ml) and without (-) benzylpenicillin.

These cultures were then shaken for 2 hr at 37°C before 10 ml samples were centrifuged (10,000 g, 10 min) and the pellets resuspended in 1 ml distilled H₂O, sonicated (4 x 20 s bursts, 20 KHz output), clarified (microfuge, 5 min) and assayed spectrophotometrically.

Assay detection limit 10^{-4} - 10^{-3} μkatal/ml.

transfer of the λ LS10 EcoRI fragment was therefore achieved by ligation of EcoRI-cleaved λ LS10 and pED815 DNA, with subsequent selection for Tc^r Cm^s transformants. As the required recombinants should constitute a high proportion of such transformants small-scale plasmid DNA preparations were made from five of them. EcoRI digestions revealed that two out of the five contained the 12 kb EcoRI fragment of λ LS10. BamHI digestions of these two recombinants demonstrated that both possessed the EcoRI fragment in the same orientation, thus making them identical, and therefore only one of them, which will henceforth be referred to as pSS10 (Fig. 4.3), was chosen for further study.

Experiments were then carried out to determine whether or not possession of pSS10 by E.coli NM259 resulted in detectable β -lactamase activity and/or a changed resistance to benzylpenicillin. As can be seen (Tables 4.1 and 4.2) both types of experiment yielded negative results.

4.6 Determination of the BamHI Restriction Map of pSS10

The sequencing of a specific gene requires not only the cloning of the gene, but also its location within the region of cloned DNA. It is known from the above results that the SP108 β -lactamase gene is present within a 12 kb cloned fragment, but the precise location of the gene within this fragment remains to be determined. One possible approach to this problem is the re-cloning of sub-fragments of the 12 kb fragment, and subsequent determination of whether or not they possess the

Table 4.2 Benzylpenicillin resistance of E.coli strains carrying recombinant plasmids.

<u>Strain</u>	<u>Benzylpenicillin concentration (µg/ml)</u>							<u>Starch plate test</u>
	0	10	20	40	60	80	100	(for β-lactamase activity)
<u>E.coli</u> NM259	+	+	-	-	-	-	-	-
<u>E.coli</u> NM259 (pED815)	+	+	-	-	-	-	-	-
<u>E.coli</u> NM259 (pSS10)	+	+	-	-	-	-	-	-
<u>E.coli</u> NM259 (pBR322)	+	+	+	+	+	+	+	+

β -lactamase gene. A prerequisite of this approach is the restriction mapping of the cloned fragment, which, in this case, was initially carried out with restriction endonuclease BamHI, the intention being to use a BamHI vector for the sub-cloning. The BamHI map of the 12 kb EcoRI fragment was determined by the BamHI mapping of pSS10. (recall, pSS10 contains the same 12 kb EcoRI fragment as λ LS10, Fig. 4.2 (a) and (b), tracks 4 and 6). An account of this will be presented here in some detail, and should be regarded as being representative of the manner in which all such restriction maps, compiled by the author, have been obtained.

This method exploits the fact that migration distances of restriction fragments of DNA, in an agarose gel, are proportional to the sizes of the fragments (McDonnell et al, 1977). It is based on a comparison of the migration distances of a series of fragments, of known size, with those of fragments whose sizes are to be determined. The fragments of known size were generated by digestion of λ cI857 DNA with: i) HindIII; ii) EcoRI/HindIII (double digest); iii) EcoRI; these digestions being represented in Fig. 4.3, tracks 2, 4 and 6 respectively. The sizes of these "standards" were based on information previously presented (Blattner et al, 1979).

The migration distances of all standard fragments, less than 10 kb in size, were plotted against log size (kb). A straight line was drawn through these points and the graph used to relate migration distance to fragment size.

Fig. 4.3 0.7% agarose gel electrophoresis of DNA fragments.

<u>Track number</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	pSS10	<u>EcoRI</u>
2	<u>λ</u> cI857	<u>HindIII</u>
3	pSS10	<u>BamHI</u>
4	<u>λ</u> cI857	<u>EcoRI</u> / <u>HindIII</u>
5	pSS10	<u>EcoRI</u> / <u>BamHI</u>
6	<u>λ</u> cI857	<u>EcoRI</u>
7	pED815	<u>EcoRI</u> / <u>BamHI</u>
8	pED815	<u>BamHI</u>
9	<u>λ</u> cI857	<u>HindIII</u>
10	pSS10	<u>EcoRI</u> /partial <u>BamHI</u>
11	pSS10	<u>EcoRI</u> /partial <u>BamHI</u>
12	<u>λ</u> 781-0	<u>BamHI</u>
13	<u>λ</u> LS10	<u>BamHI</u>
14	<u>λ</u> LS20	<u>BamHI</u>
15	<u>λ</u> NM570BV2	<u>BamHI</u>
16	<u>λ</u> cI857	<u>HindIII</u>

Notes:

- (i) Fragments in marker tracks (S) (T) and (U) are derived from HindIII digestions of λcI857 (sizes expressed in kb).
- (ii) The partially digested BamHI fragments of pSS10 (3.4 and 9.0 kb) are indicated (tracks 10 and 11).
- (iii) Tracks 13 and 14 illustrate that λLS20 contains a 5.8 kb BamHI fragment derived from λLS10.

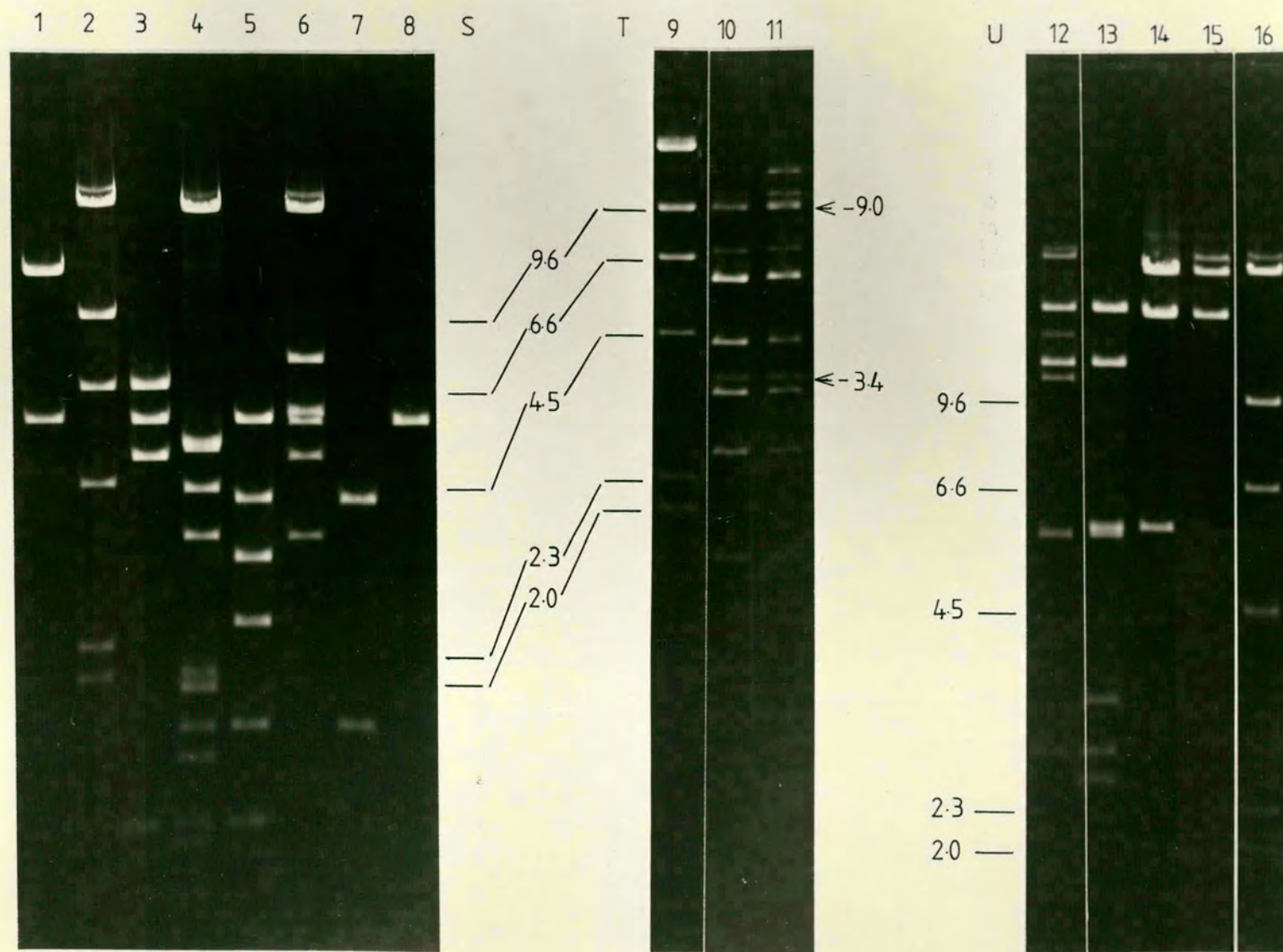


Fig. 4.3

A programmable calculator (Texas Instruments, TI 58) was used to define the straight line that optimally fits the standard coordinates. By the use of this calculator fragment size estimations can be obtained without the actual plotting, on paper, of the migration distance v log size graph. The graph (Fig. 4.4) is presented as a visual representation of the source of the information contained in Table 4.3.

Table 4.3 Restriction enzyme data for plasmids pSS10 and pED815

<u>Track (Fig.4.3)</u>	<u>Plasmid</u>	<u>Restriction enzyme(s)</u>	<u>Sizes of fragments generated (kb)</u>
1	pSS10	<u>EcoRI</u>	(12),5.7
3	pSS10	<u>BamHI</u>	6.5,5.8,4.8,1.0
5	pSS10	<u>EcoRI/BamHI</u>	5.8,4.1,3.2,2.4,1.6,1.0
7	pED815	<u>EcoRI/BamHI</u>	4.1,1.6
8	pED815	<u>BamHI</u>	5.7

Sizes quoted are ± 0.2 kb

Figures in brackets represent approximate undetermined sizes.

From this data it can be seen that pED815 has unique sites for the enzymes EcoRI and BamHI, (this is in agreement with the restriction site data of pBR325 (Bolivar, 1978)) and is 5.7 kb in size.

The data also show that pSS10 has four BamHI sites, only one of which is in the pED815 moiety of the plasmid. This indicates the existence of three BamHI sites within the inserted 12.0 kb EcoRI fragment which therefore may be used to generate subfragments of sizes 5.8, 3.2, 2.4 and 1.0 kb. It can be seen from Table 4.3 that the 3.2 and 2.3 kb subfragments are external (as these sub-fragments are not present in a single BamHI digestion of pSS10).

Fig. 4.4 Graph of log fragment size v. migration
distance on an agarose gel (see p. 63).

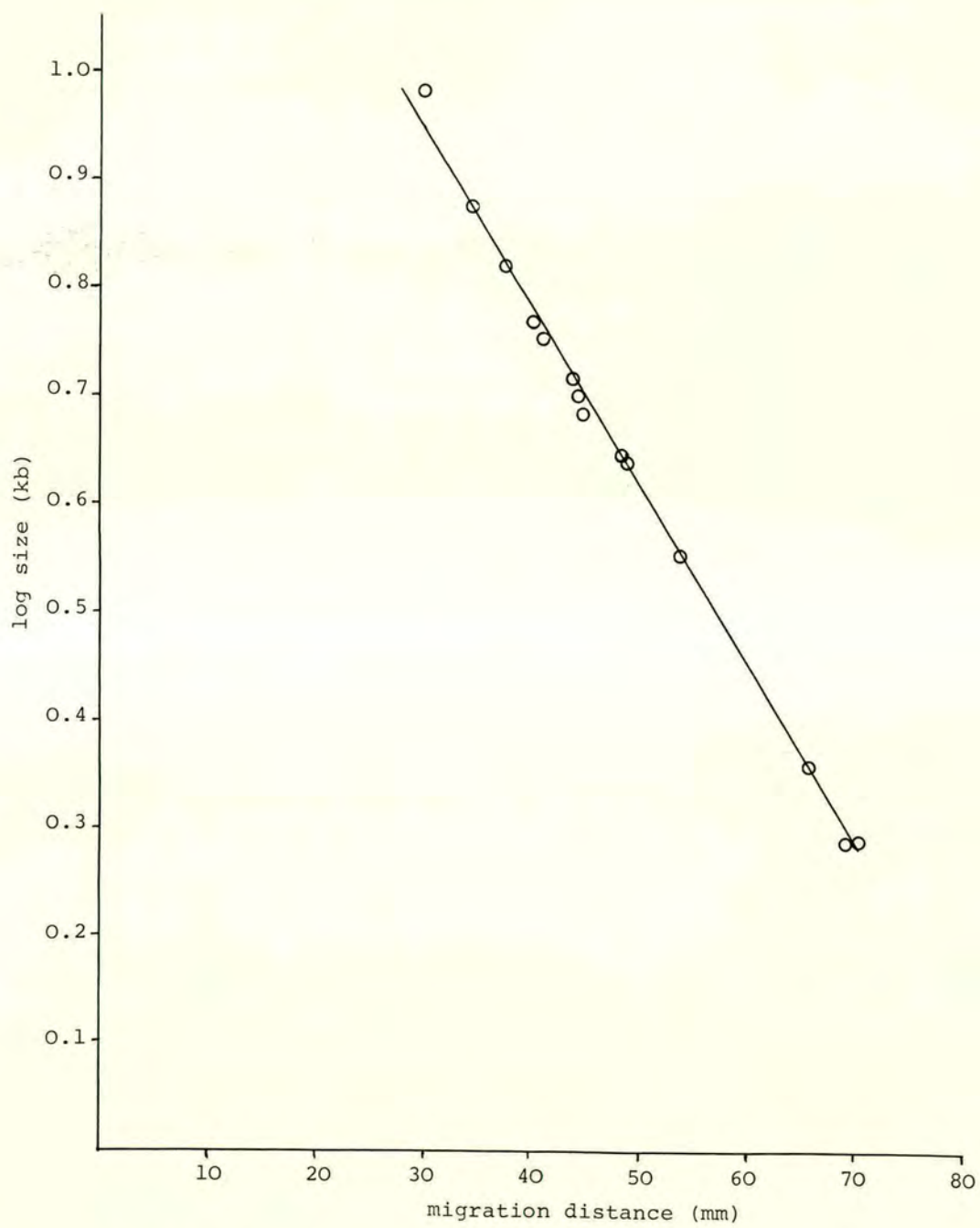


Fig. 4.4

The positions of these sub-fragments, with respect to the pED815 section of the plasmid, can also be ascertained from this data. However, the order of the internal 5.8 and 1.0 kb sub-fragments is not revealed by the information presented here, which therefore predicts two possible EcoRI/BamHI restriction maps for pSS10 (Fig. 4.5 (b) and (c)).

In order to distinguish between these two possibilities a complete EcoRI, partial BamHI digestion of pSS10 was carried out. If Fig. 4.5 (b) is correct then partially digested fragments of sizes 8.2 and 4.2 kb would be present, if however Fig. 4.5 (c), is correct then the corresponding fragments would be 9.0 and 3.4 kb in size. Fig. 4.3 (tracks 10 and 11) indicate that the latter alternative is the case and so Fig. 4.5 (c) represents the true restriction map of pSS10. Hitherto, the size of the initially cloned EcoRI fragment has been regarded as approximately 12 kb. It can be seen from Fig. 4.5 (c), however, that a more precise estimation reveals a true size of 12.4 kb. This is the size with which this fragment will in future be accorded.

4.7 Transfer of BamHI Sub-fragments of λ LS10 DNA to λ NM570BV2

As the 12.4 kb EcoRI insert of λ LS10 contains three BamHI sites (Fig. 4.5) the phage vector λ NM570BV2 (Klein and Murray, 1979) is suitable for the cloning of its sub-fragments.

Fig. 4.5 Restriction maps (EcoRI and BamHI) of pED815 and pSS10

————— vector (pED815) DNA
===== inserted (Rps.capsulata) DNA

Notes:

- (i) (b) and (c) indicate the two possible restriction maps of pSS10 predicted by Fig. 4.3, tracks 1-8.
- (ii) Fig. 4.3, tracks 9-11, indicate that (c) is the correct restriction map (see p. 65).
- (iii) The 12.4 kb EcoRI insert shown in (c) is the same as that in λ LS10.
- (iv) Distances between restriction sites are expressed in kb.

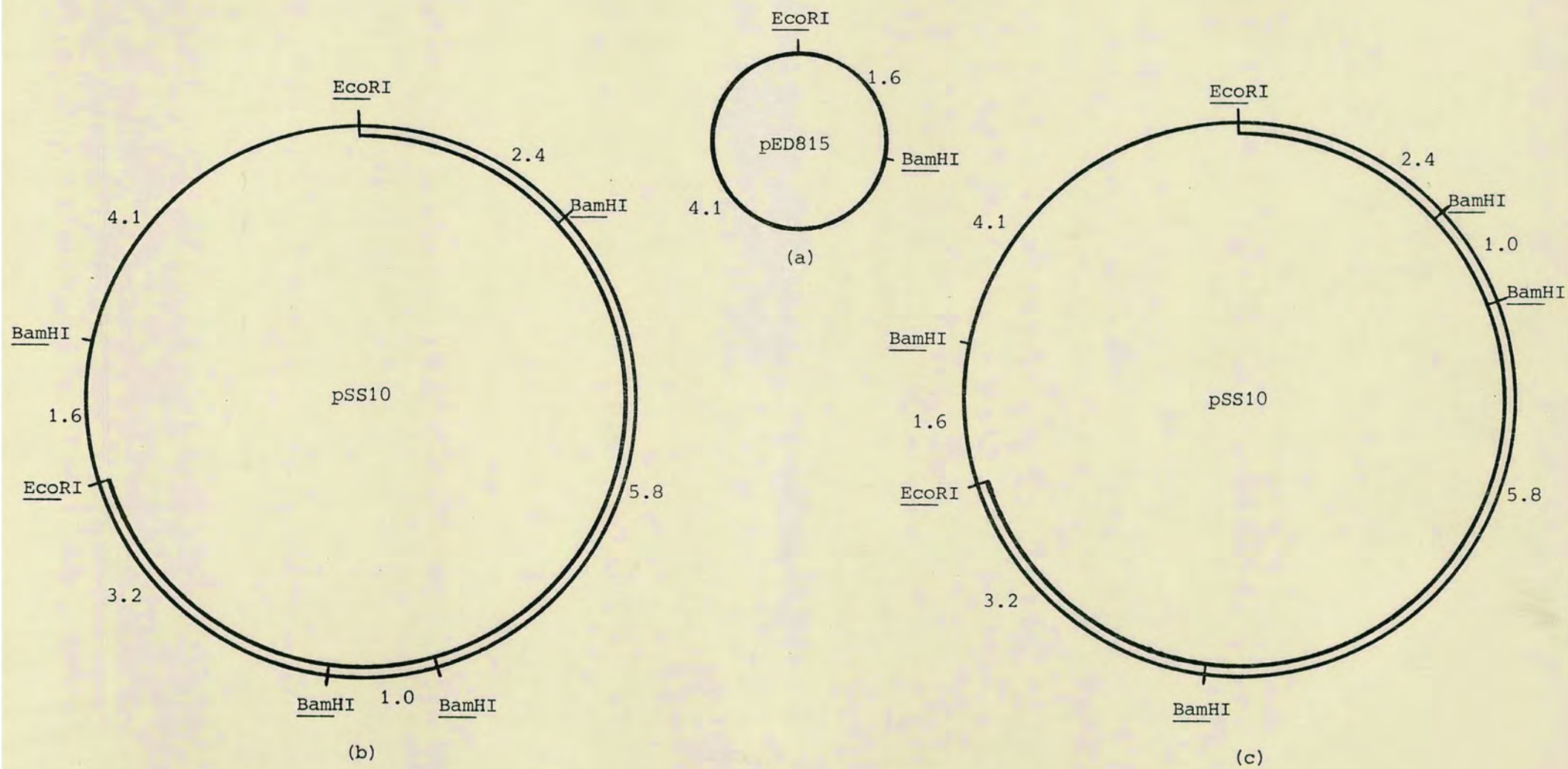


Fig. 4.5

As can be seen from Fig. 4.5 digestion of λ LS10 DNA with BamHI will generate four fragments which carry Rps.capsulata DNA. All of these fragments are a suitable size for insertion into λ NM570BV2. Following transfection of the BamHI-digested λ LS10/ λ NM570BV2 DNA ligation mixture several plaques were found, by the starch plate test, to contain β -lactamase activity. Phage from ten of these plaques were purified and plate lysate DNA preparations made from them. All of these were found, upon digestion with BamHI, to be λ NM570BV2 recombinants and most possessed two inserted λ LS10 BamHI fragments. However, the 5.8 kb fragment was common to all of them and as one recombinant contained this BamHI fragment alone it was concluded that it must carry the β -lactamase gene. The λ NM570BV2 recombinant which carries the 5.8 kb insert will be referred to as λ LS20 (Fig. 4.3, track 14).

In order to confirm that λ LS20 carries the Rps.capsulata SP108 β -lactamase structural gene liquid lysates produced by it and λ NM570BV2 were treated as described in Section 4.4 above. Samples from each were then run on an isoelectric focusing gel. Fig. 4.6 illustrates that the λ LS20 sample produced two bands of β -lactamase activity on this gel which were identical to those produced by strain SP108. The λ NM570BV2 sample exhibited no such activity. The lysates were also subjected to spectrophotometric assays of β -lactamase activity, the results of which are presented below in Table 4.4.

Fig. 4.6 Isoelectric focusing of the β -lactamase encoded by λ LS20 (p. 66).

<u>Tracks number</u>	<u>Sample</u>
1	cytochrome c-551 (marker)
2	<u>Rps.capsulata</u> SP108 cellular sonicate
3	Liquid lysate of <u>E.coli</u> NM259 infected with λ NM570BV2
4	Liquid lysate of <u>E.coli</u> NM259 infected with λ LS20
5	azurin (marker)

Isoelectric focusing was carried out in an agarose gel and the pH gradient generated by pH 4.0-6.5 Ampholines (LKB) (see p. 45).

Note:

- (i) It can be seen (track 3) that no β -lactamase activity was detectable in the lysate produced by λ NM570BV2, but that β -lactamase activity did exist in the lysate produced by λ LS20 (track 4). Furthermore, the β -lactamase activity of λ LS20 corresponds to that of Rps.capsulata SP108 (track 2).

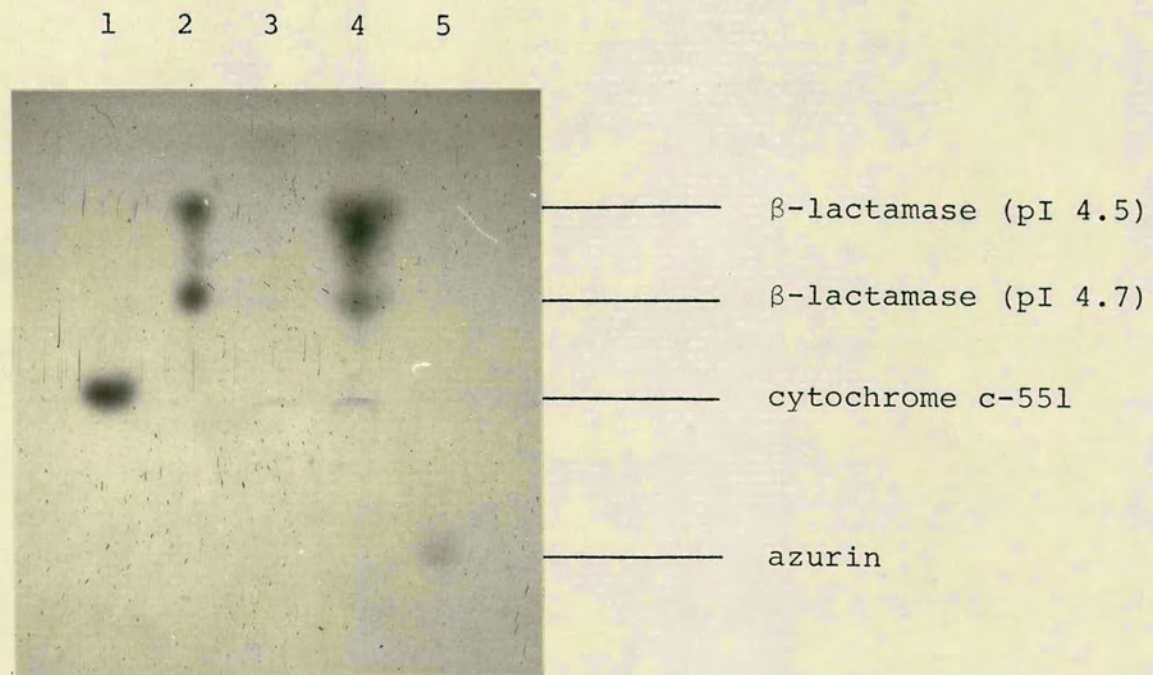


Fig. 4.6

Table 4.4 β -Lactamase production by λ NM570BV2 and λ LS10

<u>Host</u>	<u>Phage</u>	<u>β-lactamase activity</u>
		(μ katal/ml lysate supernatant)
<u>E.coli</u> NM259	λ NM570BV2	none detectable
<u>E.coli</u> NM259	λ LS10	8.1×10^{-3}

Assay detection limit 10^{-4} - 10^{-3} μ katal/ml.

4.8 Location of the *Rps.capsulata* β -Lactamase Gene in λ LS20

In an attempt to define more precisely the position of the β -lactamase gene in the 5.8 kb BamHI fragment, a BglIII restriction map of it and adjacent areas of λ LS20 was made (Fig. 4.8(a)).

The restriction endonuclease BglIII recognises the hexanucleotide sequence 5'-A-G-A-T-C-T-3', which is cut between the 5'-A and G residues thus forming fragments with the 5' single stranded nucleotide projection 5'-G-A-T-C-3'. As this cohesive end is identical to that formed by the cutting of the hexanucleotide sequence 5'-G-G-A-T-C-C-3' with BamHI, BglIII-generated restriction fragments may be ligated with BamHI-generated fragments. This makes it possible to clone BglIII or BglIII/BamHI restriction fragments in BamHI cloning vectors, such as λ NM570BV2.

Fig. 4.8 (a) indicates that a double digestion of λ LS20 DNA with BamHI and BglIII would generate three restriction fragments which comprise Rps.capsulata DNA. Insertion of a single, or two combined, Rps.capsulata DNA fragments into the vector λ NM570BV2 should permit a more precise location of the β -lactamase gene. In order to

Fig. 4.7 0.7% agarose gel electrophoresis of DNA fragments.

<u>Track number</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	λ NM57OBV2	<u>Bam</u> HI/ <u>Bgl</u> II
2	λ LS20	<u>Bam</u> HI/ <u>Bgl</u> II
3	λ NM57OBV2	<u>Eco</u> RI/ <u>Bam</u> HI
4	λ LS20	<u>Eco</u> RI/ <u>Bam</u> HI
5	λ LS20	<u>Bgl</u> II
6	λ LS20	<u>Eco</u> RI/ <u>Bgl</u> II
7	λ cI857	<u>Hind</u> III
8	λ NM57OBV2	<u>Eco</u> RI
9	λ LS20	<u>Eco</u> RI
10	λ LS21	<u>Eco</u> RI
11	λ LS22	<u>Eco</u> RI
12	λ cI857	<u>Hind</u> III
13	λ LS20	<u>Eco</u> RI/ <u>Bam</u> HI
14	λ LS21	<u>Eco</u> RI/ <u>Bam</u> HI
15	λ LS22	<u>Eco</u> RI/ <u>Bam</u> HI

Notes:

- (i) Fragments in marker tracks (S) and (T) are derived from HindIII digestions of λ cI857 (sizes expressed in kb).
- (ii) Tracks 14 and 15 indicate the different orientations of the 4.9 kb (2.3 kb plus 2.6 kb) inserts of λ LS21 and λ LS22.

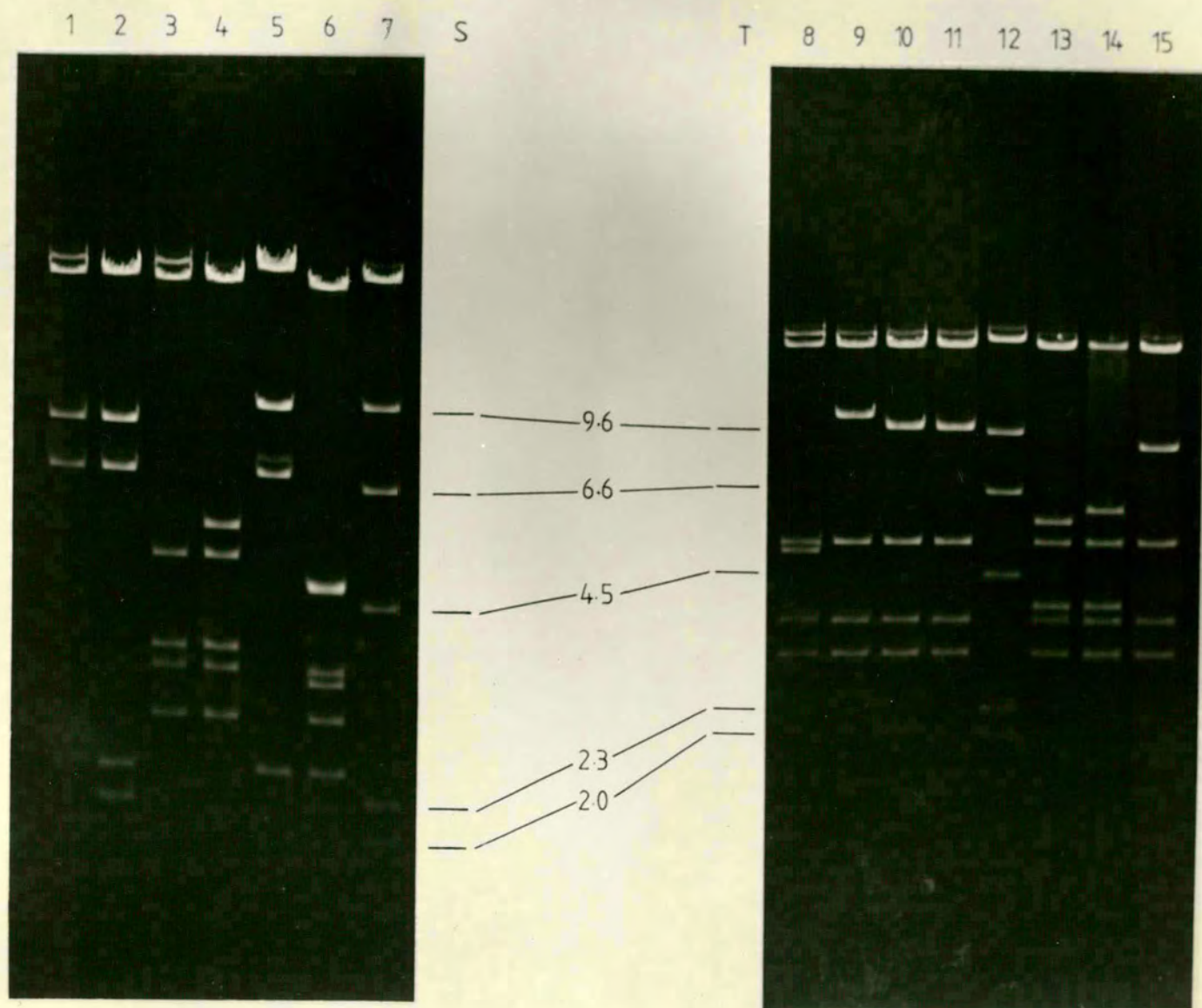
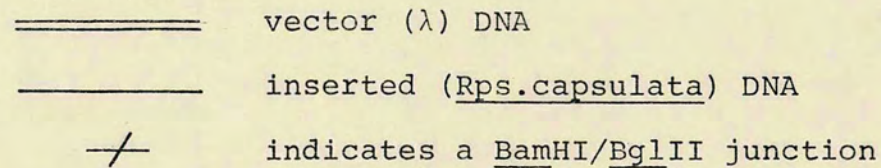


Fig. 47

Fig. 4.8 Restriction maps (BamHI and BglII) of the inserted fragments and adjacent regions of λ LS20-22



Notes:

- (i) The maps presented are based on data derived from Fig. 4.7 and Fig. 4.3, tracks 12-16.
- (ii) Inserts of λ LS21 and λ LS22, both of which have lost the 0.9 kb BamHI/BglII fragment of λ LS20, are present in opposite orientations.
- (iii) Distances between restriction sites are expressed in kb.

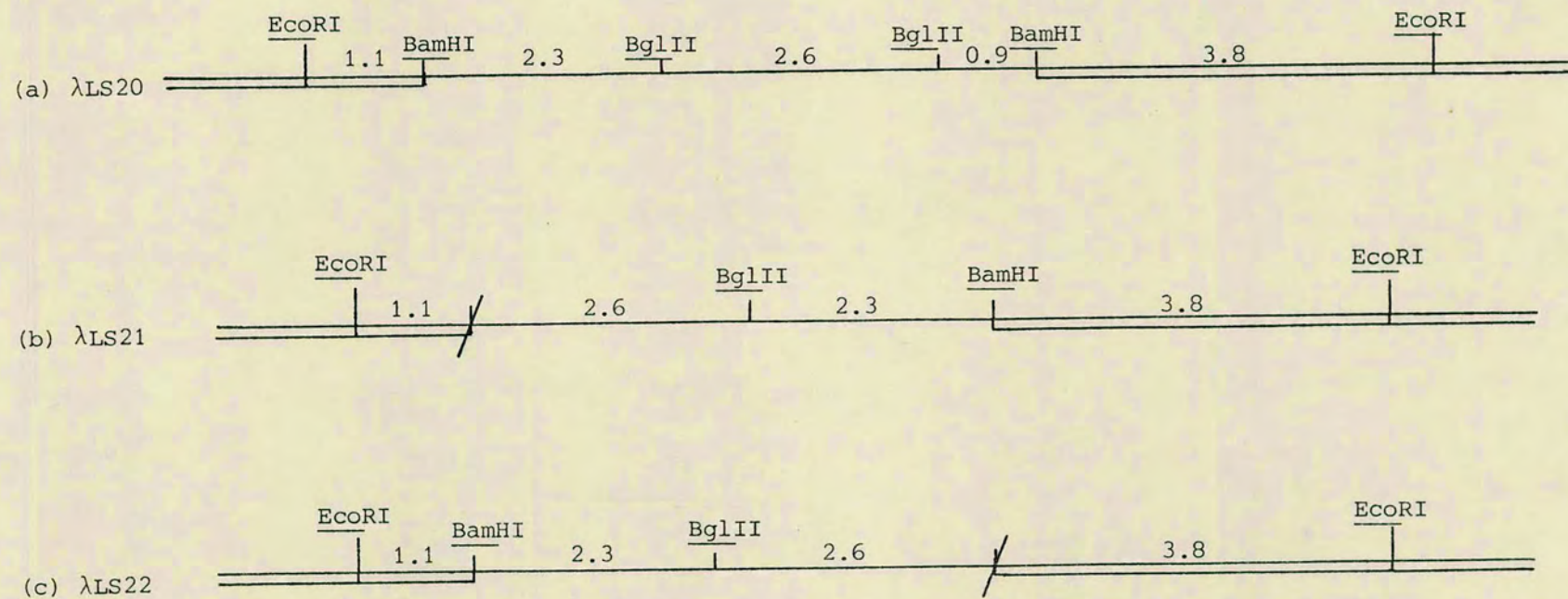


Fig. 4.8

effect this transfer the following ligation (LA) and control ligation (LC) were set up.

Ligation LA contained: i) λ NM570BV2 DNA cut with BamHI
ii) λ LS20 DNA cut with BamHI and BglII
Ligation LC contained: i) λ NM570BV2 DNA cut with BamHI
ii) λ LS20 DNA cut with BamHI and BglII
iii) λ LS20 DNA cut with BamHI

Both ligations were carried out in equal volumes and each had equivalent concentrations of the left and right arms of λ NM570BV2 vector DNA. The major difference between the two ligations was that LC contained the 5.8 kb BamHI fragment from λ LS20 and therefore definitely possessed the β -lactamase gene on a single insertable restriction fragment. It may be, however, that the β -lactamase gene is cut by one of the two BglII sites which cleave the Rps.capsulata segment of DNA in λ LS20. This would therefore mean that no single restriction fragment present in ligation LA possessed an intact β -lactamase gene. If so, a β -lactamase-producing recombinant could only have emerged from ligation LA after the ligation, in the correct orientation, of two, or more, restriction fragments which carry a section of the β -lactamase gene, and the subsequent insertion of this combination into the vector λ NM570BV2. This is an event which would occur far less frequently than the relatively simple insertion of the 5.8 kb BamHI fragment, present in ligation LC. Therefore, if the β -lactamase gene contains a BglII site, this should have been reflected by the appearance, of β -lactamase-containing recombinants, at a much lower frequency in

the transfections of ligation LA, compared to ligation LC.

It is necessary to state that the concentration of the 5.8 kb fragment, and each of the three smaller Rps.capsulata DNA restriction fragments, in ligation LC, was approximately one half of that of the three Rps.capsulata DNA fragments in ligation LA. Therefore, if the β -lactamase gene is not cut by the enzyme BglII, and is thus present on only one of these fragments, β -lactamase-producing recombinants^{would}/have been expected to appear at approximately the same frequency from both ligations.

Samples of competent cells were transfected with 50 μ l of each of the ligation solutions, with the following results:

Table 4.5 Transfection of ligations LA and LC

	<u>Ligation</u>	
	<u>LA</u>	<u>LC</u>
Total no. of transfectants obtained	7,000	10,000
% Phage producing β -lactamase (starch plate test).	0.1	2.7

The seven β -lactamase-producing recombinants, which arose from ligation LA, were purified, and plate lysate DNA preparations made from them. Restriction enzyme analyses, of DNA from these phage, demonstrated that all of them had lost the 0.9 kb BamHI/BglII fragment of λ LS20, but had retained the other two Rps.capsulata DNA fragments (2.3 kb BglII and 2.6 BamHI/BglII) of this phage.

Furthermore, the two fragments that were retained were present in the same orientation, with respect to one another, in all of the phage, but were present, amongst the seven recombinants, in both possible orientations with respect to vector DNA. Single examples, of phage representing both orientations, were taken, and referred to as λ LS21 and λ LS22 (Fig. 4.8 (b) and (c)).

CHAPTER 5

It is a capital mistake to theorise
before one has data.

Scandal in Bohemia

Doyle, Sir Arthur Conan (1859-1930)

Discussion

The MIC values for benzylpenicillin (Table 3.1) confirm the previous reports (Weaver et al, 1975; J.D. Wall, personal communication) which describe the marked resistance of strain SP108 and the relative sensitivity of strain SP109 to this antibiotic. However, strain SP109 does appear to possess a greater resistance to benzylpenicillin than strain St. Louis, although a quantitative assessment of this requires further measurements.

Starch plate detection of β -lactamase activity (Figs. 3.1 and 3.2) and isoelectric focusing studies (Fig. 3.4) suggest that simply the possession of a β -lactamase by SP108 is insufficient to account for the high penicillin-resistance of this strain, as SP109 is shown to produce the same type of β -lactamase.

Induction studies indicate that increased β -lactamase production contributes to the penicillin-resistance of SP108. The inducibility of the β -lactamase of this strain results in the synthesis of much larger amounts of the active enzyme than is possible for strain SP109 (Table 3.2 and Fig. 3.3). It is possible, however, that the permeability barrier of strain SP108, the existence of which is indicated by a crypticity value of

10 for this strain with benzylpenicillin (Section 3.4), may also play a role in resistance to penicillin. As the penicillin-sensitivity of strain SP109 correlates an inability to produce large amounts of β -lactamase any role played by the permeability barrier is probably small compared to that of the β -lactamase.

Maximal induction of the SP108 β -lactamase with benzylpenicillin (100 μ g/ml) occurs approximately 100 min after addition of the inducer. This prolonged response may be partially due to the permeability barrier, which, it has been suggested (Nordstrom and Sykes, 1974), is responsible for the lag period that occurs on induction of the Ps.aeruginosa Sabath and Abraham enzyme.

The B.licheniformis β -lactamase also exhibits a slow induction and this was contrasted, by Collins (1979), to the induction of β -galactosidase in E.coli. Inducers of β -galactosidase are, however, able to penetrate the cytoplasmic membrane, whereas penicillin is not thought to do so and must therefore exert its effect on induction from an extra-cytoplasmic location. Induction from such a location must be a more complex phenomenon than when inducer is present in the cytoplasm and this may also contribute to the slow induction of β -lactamases.

Release by osmotic shock has been regarded as being indicative of a periplasmic location for the enzyme concerned. However, Watson (1980) has shown that a certain portion of the osmotic shock releasable TEM-type β -lactamase is present in the outer membrane of E.coli.

This suggests that it is more appropriate to regard this type of release as indicating a "surface" location. Furthermore, Smith and Wyatt (1974) stated that release by osmotic shock is not simply a function of enzyme location, but is also dependent upon molecular weight. These authors observed that β -lactamases with a molecular weight of about 20,000 were released by osmotic shock but those with molecular weights of 30,000, or more, were retained by a variety of Gram-negative bacteria during osmotic shock treatment.

Table 3.4 suggests that the β -lactamase is released from both induced and uninduced strains of SP108 by the cold osmotic shock procedure. Assuming that all cells produce an equivalent amount of enzyme, insufficient cell lysis (a maximum of 30% as indicated by viable cell counts (Table 3.5)) occurred to account for the large amount (57%) of enzyme released. Thus, although a cytoplasmic enzyme assay was not included as a control to indicate cell lysis it does not appear that lysis was responsible for the release of β -lactamase. It therefore seems that at least some of the SP108 β -lactamase has a surface location.

Isoelectric focusing of β -lactamase samples, from strains SP108 and SP109, produced bands of activity at positions corresponding to pI values of 4.5 and 4.7 in each case (Fig. 3.4). A similar result was obtained when wider range Ampholines (pH 3.5-10.0) were used to generate the pH gradient. This shows that both strains produce the same type of β -lactamase activity and,

furthermore, as these pI values do not correspond to any of those previously published for β -lactamases (Sykes and Matthew, 1976), that the SP108/SP109 enzyme may be regarded as being "novel". The two bands of β -lactamase activity produced by each sample could be due to the presence of two distinct β -lactamases in each strain. An alternative explanation is that the two bands represent different forms of the same enzyme. It is known that Gram-negative β -lactamases are secreted into the periplasm and that this requires the processing of the initial gene product, thus giving rise to different forms of the same enzyme. Support for this type of processing being responsible for the two focused bands comes from the isoelectric focusing of an osmotic shock released sample of this enzyme. This sample, although still producing both bands of activity was greatly enriched for the pI 4.5 species (Fig. 3.4). The osmotic shock released sample would be expected to be enriched for the processed or extracellular form of the enzyme, which may therefore be represented by the activity with an isoelectric point of 4.5.

Throughout this discussion it will be assumed that the processing of a single enzyme is responsible for the two distinct bands present after isoelectric focusing. It is appreciated, however, that this has not been conclusively proven.

Substrate profile determinations are often carried out on crude enzyme preparations which may contain more than one type of β -lactamase. The profile presented in

Table 5.1 Characteristics of the TEM-type β -lactamase encoded by plasmid RPl in E.coli.

The data presented is derived from Sykes and Matthew (1976).

	<u>Ampicillin</u>	<u>Cephaloridine</u>
Rate of hydrolysis (relative to benzylpenicillin)	150	120
Km	40	800
Vmax	1,500	1,500
Crypticity	40	1
Single cell resistance ($\mu\text{g/ml}$)	2,500	2

Table 3.3, although determined with such a crude preparation, is thought unlikely to have contained significant amounts of any second type of β -lactamase as isoelectric focusing did not reveal any such activity.

Relative rates of hydrolysis, as presented in substrate profiles, do not reflect their dependence upon the kinetic parameters of the enzyme concerned (Table 5.1). Furthermore, as the permeability properties of the outer membrane affect both crypticity values and single cell resistances these values also cannot be related to substrate profile figures (Table 5.1).

However, as substrate profiles for a given enzyme seem to be independent of the replicon specifying it, or the bacterial species in which it is present, they are of value in determining whether the enzyme is a penicillinase, cephalosporinase, or a broad-spectrum β -lactamase.

The substrate profile presented in Table 3.3 indicates that the SP108 β -lactamase is a penicillinase and, as this profile does not resemble any that have been previously published, supports the isoelectric focusing evidence indicating that this is a novel enzyme.

The presence of four distinct classes of plasmids in strains SP108 and SP109 makes it necessary to consider the possibility of a plasmid location for the β -lactamase specified by these strains.

Several different classes of plasmids have now been isolated from the genus Rhodopseudomonas (Table 5.2),

Table 5.2 Plasmids isolated from the genus Rhodopseudomonas.

<u>Bacterial strain</u>	<u>No. plasmids</u>	<u>Plasmid sizes (kb)</u>	<u>Reference</u>
<u>Rps.sphaeroides</u> NC1B 8327	2	Both plasmids in range 105-112	Gibson and Niederman (1970)
<u>Rps.sphaeroides</u> 2.4.1	3	112, 99 and 42 (51)	Saunders <u>et al</u> (1976)
<u>Rps.capsulata</u> BH9	2	141 and 111	Hu and Marrs (1979)
<u>Rps.capsulata</u> SP108	4	127, 92, 15 and 8.4 (11.4)	This work.
<u>Rps.capsulata</u> SP109	4	127, 92, 15 and 8.4	This work.
<u>Rps.capsulata</u> St. Louis	1	78	This work.

Figures in parentheses indicate observed size changes.

and although their sizes suggest that certain of them may be identical no direct evidence for this has yet been obtained. There does not, however, appear to be any similarity between the Rps.capsulata St. Louis plasmid and those of strains SP108 and SP109. Firstly, as their sizes are different and secondly as restriction patterns of plasmid preparations from SP108 and SP109 show no similarities with that of the St. Louis strain. If these plasmids do contribute an indispensable function(s) it is likely that they would be related and therefore possess conserved sequence similarities. As these have not been detected, at least for the plasmids studied here, there is no evidence in support of an indispensable function for the plasmids of Rps.capsulata.

The susceptibility of many rhodopseudomonads to bacteriophages indicates a possible role for the plasmids found in this group. One of the best studied rhodopseudomonad bacteriophages is RØ6P (Tucker and Pemberton, 1978). This is a temperate bacteriophage which plaques on strains of Rps.sphaeroides. The encapsidated phage genome occurs as a supercoiled, circular, DNA duplex, 33 Md (50 kb) in size. Surprisingly, the phage genome seems to exist as a dimeric plasmid in the lysogenic cell. A further interesting characteristic of this phage is that it carries a penicillinase gene, thus cells which are lysogenic for RØ6P are penicillin-resistant.

The specific susceptibility of many Rps.capsulata strains to bacteriophage, certain of which have been shown

to exhibit pseudolysogeny (Wall et al, 1975), raises the possibility that certain of the plasmids of this species may represent phage genomes.

Bacteriocin production is known to be plasmid-mediated in many Gram-negative species. It is therefore possible that the plasmids of Rps.capsulata, several strains of which have been shown to produce bacteriocins (Wall et al, 1975), are involved with this function.

Identical restriction patterns are formed by the SP108 and SP109 plasmids on digestion with the same restriction endonucleases (Fig. 3.5). This confirms the electron microscopic data indicating that the same plasmid complement is present in both strains (Table 3.6).

Perlman and Rownd (1975) observed that the R-factor NR1 could respond to the presence of chloramphenicol in the culture medium by increasing its number of copies of the segment of DNA which carried the chloramphenicol-resistance gene (the r-determinant). This phenomenon is referred to as the transition of NR1. It was further observed that during subsequent growth in drug-free medium the extra copies of the r-determinant were lost. This resulted in NR1 regaining its original size.

The restriction patterns of SP108 plasmids appear to be unaffected by the presence of benzylpenicillin in the culture medium (Fig. 3.5). It does not seem, therefore, that a plasmid-borne gene is being increased in copy-number, in a manner analogous to the transition phenomenon, in order to contribute to, or effect, an increased β -lactamase production or penicillin-

resistance of this strain.

The increase in size observed for the smallest class of plasmid in strain SP108 (Fig. 3.7) may have arisen by one of two types of mechanism. Firstly, an intramolecular duplication/multiplication event, similar to those observed by Perlman and Rownd, may have occurred, resulting in the repetition of a segment of DNA present in the 8.4 kb plasmid. Alternatively, the size increase may have been due to the insertion, into the 8.4 kb plasmid, of a segment of DNA previously existing in a different replicon. Transposable segments of DNA, capable of this type of insertion, have been recently reviewed (Calos and Miller, 1980).

Electron microscopic evidence indicates that the 8.4 kb plasmid does exist in the SP108-1 plasmid preparation, albeit less frequently than the 11.4 kb plasmid (data not shown).

It is not known whether the 11.4 kb plasmid existed in the single cell which gave rise to the culture from which the SP108-1 plasmid preparation was derived. Nor are the copy-numbers of these plasmids known. Thus, it is not possible to speculate effectively about whether or not the 11.4 kb plasmid confers any selective advantage on its host cell. However, no selection was knowingly applied during growth of the SP108-1 culture.

Plasmid encoded functions often exhibit a marked instability which results from loss of the plasmid by growing cells. The rate of loss can be increased by

the incorporation, into the growth medium, of substances such as SDS or acridine orange, which are referred to as curing agents. Such a curing agent-induced increase in the rate of loss is therefore indicative of a plasmid location for the function concerned.

Weaver et al (1975) stated that strain SP108 produced penicillin-sensitive clones at a high frequency (about 1% of colonies on streaked plates). However, no such spontaneously occurring sensitive isolates were obtained during the course of this work (Section 4.1). Furthermore, attempts to cure strain SP108 of penicillin-resistance, with a proven curing agent (EtBr) (Bouanchaud et al, 1969) were also unsuccessful. Thus, no evidence regarding the location of the SP108 β -lactamase gene can be derived from these studies.

The ability of recombinant phages λ LS10 and λ LS20 to produce a β -lactamase that focuses on an isoelectric focusing gel (Fig. 4.6) at the same positions as the SP108 β -lactamase demonstrates that λ LS10 must have acquired a fragment of Rps.capsulata SP108 DNA which carries the β -lactamase structural gene. Proof that the 12.4 kb insert of λ LS10 derives from the genome of Rps.capsulata SP108 was obtained by hybridisation of λ LS10 DNA against an EcoRI digestion of SP108 plasmid DNA. Fig. 4.2 shows that the insert corresponds to a 12.4 kb EcoRI fragment of SP108 and SP109 plasmid DNA. λ LS10 DNA was not observed to hybridise to EcoRI-digested SP108 total genomic DNA (data not presented), despite the fact that such a preparation would be expected to contain plasmid

DNA. However, this is probably because plasmid DNA constitutes only a fraction of total genomic DNA, thus making hybridisation, using a plasmid directed probe, to a genomic DNA preparation more difficult. The results presented in Fig. 4.2 therefore indicate that the β -lactamase of strain SP108 is a plasmid-mediated enzyme. There have been no previous reports of a plasmid-mediated, inducible β -lactamase in a Gram-negative organism.

The fact that both the pI 4.5 and pI4.7 species of β -lactamase activity can be cloned on a single EcoRI fragment supports the view that these are the activities of a single gene.

As the β -lactamase structural gene is present on a 12.4 kb EcoRI fragment it cannot reside on the smallest (8.4 kb) plasmid possessed by strain SP108.

Plasmid pBR322 carries a TEM-type β -lactamase and thus any hybridisation between λ LS10 and pBR322 DNA may have been due to homology between the different β -lactamase genes possessed by each. The inability to demonstrate such homology (Fig. 4.2), however, does not necessarily suggest that it does not exist as Brammar et al (1980) were unable to demonstrate hybridisation of DNA sequences known to code for homologous β -lactamases.

As mentioned above Weaver et al (1975) were able to isolate penicillin-sensitive derivatives of strain SP108 at a high frequency, but studies described in this work were not able to detect a similar instability. The high rate of loss described by Weaver et al is indicative of a plasmid-mediated resistance gene, an indication that has

now been substantiated by the hybridisation studies also described above. It is surprising therefore to find that a supposedly representative, sensitive strain (SP109), supplied by Weaver's laboratory, has not acquired penicillin-sensitivity by plasmid loss. Such observations cast doubt on the nature of the Pen^r phenotypes of the SP108 derivatives obtained by Weaver et al.

It is necessary to state that J.D. Wall (personal communication) has informed us that strain SP109 may have been a contaminant present in the initial isolation of strain SP108. This is consistent with the isolation by Weaver et al of penicillin-sensitive derivatives and our inability to repeat this, as the latter work was carried out on a purified penicillin-resistant cell line.

Other evidence, however, such as the possession by strains SP108 and SP109 of identical plasmid complements, and the same type of β -lactamase, suggests that SP109 is a derivative of SP108. Indeed, the only differences known to exist between the two strains are the basal level of synthesis and inducibility of the β -lactamase (Fig. 3.3), differences which may have resulted from a single mutational event. Such a mutation is unlikely to revert spontaneously at a frequency greater than 1×10^{-8} per bacterium per generation, and therefore would not be expected to have been detected in the experiment described above (Section 4.2).

It is possible to detect SP108 β -lactamase activity when the enzyme's structural gene is present in a phage vector (λ LS10), but not when it is present, on the same

EcoRI fragment, in a plasmid vector (pSS10) (Tables 4.1 and 4.2). This suggests that, in the latter system, either the gene is not being expressed or its product is not normally processed. Lack of processing may result in the gene/^{product}remaining cytoplasmic and thus being unable to effect penicillin-resistance.

Bruce and Warren (1979) presented evidence which indicated that the RPl TEM-type β -lactamase when present in Pseudomonas acidovorans was expressed but that the gene product remained in the cytoplasm. Furthermore, the enzyme did not confer any resistance to penicillin, a fact that was attributed to the lack of secretion.

The absence of a Pen^r phenotype in strains of Rps.sphaeroides carrying R-factor R68.45 has been previously discussed (Section 1.2d). It may be that this lack of resistance is also due to the retention of the β -lactamase concerned. It should be recalled that the other antibiotic resistances carried by R68.45 (kanamycin and tetracycline), which are effected by intracytoplasmic gene products, were expressed normally in Rps.sphaeroides.

Let us maintain the assumption that the pI 4.5 and pI 4.7 SP108 β -lactamase bands are products of a single gene, and further assume that the pI 4.5 band represents the secreted form of the enzyme, as was suggested above. The fact that both forms of this enzyme are produced in an E.coli host by λ LS20 (Fig. 4.6) must then indicate that processing of the β -lactamase can occur in E.coli. This in turn would suggest that the inability of a

plasmid-mediated SP108 β -lactamase gene to confer resistance is due to its non-expression. The inability to detect cytoplasmic SP108 β -lactamase activity in E.coli (pSS10) (Table 4.1) supports the expression-problem hypothesis.

If a problem of expression does exist for the SP108 β -lactamase gene in pSS10, then it is probably expressed by phage promoters in λ LS20. Phage promoters which efficiently transcribe fragments of DNA inserted into the lambda genome have been extensively exploited (Davison et al, 1974). It is possible to determine whether or not expression of genes, present on inserted fragments, are transcribed from phage promoters by studying their expression under conditions in which transcription from these promoters is prevented (Jaskunas et al, 1977). This type of experiment involves infection, with the recombinant phage, of a lysogenic host strain that has been exposed to a dose of U/V irradiation sufficient to prevent further expression of all genomic functions. Such an experiment would determine whether or not promoters present on the cloned fragment were being used to initiate transcription of that fragment, or whether this transcription was dependent upon a phage promoter(s).

There are several circumstances in which a cloned gene may encounter problems of expression. The first of these is that the structural gene may have been separated from its own promoter during the cloning procedure. I believe this to be unlikely in the case of SP108 β -lactamase as the gene occupies a central position

in the cloned fragment present in vectors λ LS10 and pSS10 (Fig. 4.5). Secondly the possibility exists that the genes own promoter is not efficiently recognised by the host cell's RNA polymerase. The kanamycin and tetracycline resistances of RP4 are normally expressed in Rps.sphaeroides, indicating that the RNA polymerase of this species recognises the promoters of these resistance genes. This suggests that no major expressional barrier exists between the Rhodopseudomonas and Escherichia genera.

The relative frequencies with which penicillinase-producing recombinant phage are obtained from ligations A and C of Section 4.8 suggest that a BglIII target site exists within, or near to, the SP108 β -lactamase structural gene. The fact that all penicillinase-producing phage, derived from ligation A, are recombinants, and carry only two Rps.capsulata fragments (the 2.3 kb BglIII and 2.6 kb BamHI/BglIII fragments of λ LS20) which are present, in all cases, in the same orientation relative to one another, further supports this view. This observation also suggests that the BglIII site concerned is that which defines the junction of these two fragments (Fig. 4.8).

Further evidence for this BglIII site marking the location of the SP108 β -lactamase gene could be obtained by the insertion of restriction fragments into this site. Such insertions would, if this is the site of the gene, be expected to disrupt β -lactamase synthesis. The separate cloning of the 2.3 and 2.6 kb fragments in

λ NM570BV2 would, however, provide a more decisive conclusion to these considerations. Both of these approaches are experimentally feasible.

Aiking and Sojka (1979) commented that the photosynthetic bacteria were unusually well suited for research related to energy conservation. They also stated that the increased interest in these organisms is partly due to the variety of energy transduction systems and resultant modes of growth that they exhibit. Madigan and Gest (1979) stated that Rps.capsulata was capable of multiplying in at least five different physiological growth modes, and suggested that this species is one of the most metabolically versatile prokaryotes known. The growth modes described by these authors included chemoautotrophic growth, which was carried out under aerobic conditions, in darkness, and found to be strictly dependent on H_2 , O_2 and CO_2 as the sole carbon source. Apart from utilising molecular hydrogen in chemoautotrophic growth Rps.capsulata can also produce it (Jouanneau et al, 1980), a function that is related to the nitrogen fixing capabilities of this species (Colbeau et al, 1980).

The most attractive characteristic of the rhodopseudomonads to the research scientist, however, continues to be their photosynthetic ability. Most of the research that has been carried out on Rps.capsulata has been directed towards an understanding of this process (Marrs and Gest, 1973; La Monica and Marrs, 1976).

Saunders (1978) stated that although these types of study have led to a considerable accumulation of biochemical and biophysical data concerning the mechanisms of bacterial photosynthesis, there has, until recently, been a marked lack of complementary genetic information. The discovery of the GTA permitted the first genetic analysis of Rps.capsulata (Marrs, 1974), but the author believes (Marrs et al, 1977) that the application of this system is limited by the size of DNA segment that can be transferred.

Although Sistrom (1977) demonstrated the transfer of Rps.sphaeroides chromosomal genes by the conjugative plasmid R68.45, the low frequency with which this occurred prompted Tucker and Pemberton (1979) to suggest that the system may be of limited value. These authors, in an attempt to improve the efficiency of this type of system, have inserted the temperate bacteriophage Mu into plasmid RP4, with the hope that this will promote "in vivo genetic engineering" in Rps.sphaeroides.

One of the most powerful techniques to have been introduced into molecular biology in recent years is that of in vitro genetic manipulation by the use of restriction endonucleases. This technique has been used several times during the work described in this thesis and so practical details of the technique have already been presented. The ability to apply this type of genetic manipulation to the rhodopseudomonads would undoubtedly enhance the groups' research status by partially compensating for the absence of naturally

occurring systems of conjugation and transduction. The two main prerequisites for the establishment of this type of system are firstly an ability to transform the species in question, and secondly the existence of a suitable vector molecule.

Tucker and Pemberton (1980) have recently described the successful transformation of Rps.sphaeroides with RØ6P DNA. This indicates that the development of an in vitro genetic manipulation, or molecular cloning, system in the rhodopseudomonads may be dependent upon the finding, or construction, of a suitable vector. Such a vector molecule would be required to be capable of self-replication and preferably exist in many copies in the cell. Plasmids or phages that are native to the rhodopseudomonads are natural candidates as they are likely to exist stably in these organisms. None of the phages of this group are, as yet, sufficiently well characterised to be seriously considered, and most of the plasmids are too large in size to be efficiently transformed.

The two known small plasmids are the 15.0 kb and 8.4 kb plasmids isolated during the course of this work. It is already known that the 8.4 kb plasmid, which may be a multi-copy plasmid, satisfies a further requirement of vector molecules by the possession of unique target sites for three commonly used restriction enzymes (Fig. 3.7(a)). Although the 8.4 kb plasmid is not known to confer any readily recognisable phenotype upon its host cell it would be perfectly feasible to insert an

SP108 β -lactamase-carrying fragment into this plasmid. This may then permit Rhodopseudomonas species, transformed with the recombinant plasmid, to be detected by virtue of their resistance to penicillin. The quality and efficiency of E.coli plasmid vectors have been considerably improved by this type of construction (Bolivar, 1978).

An important potential application of molecular cloning is the overproduction by bacteria of certain gene products, some of which may not normally be synthesised in prokaryotic cells. For example, Nagata et al (1980) constructed a recombinant plasmid which, when present in E.coli, is capable of directing the synthesis of a polypeptide with human leukocyte interferon activity. It is hoped that this type of production will facilitate the purification of such gene products, and, if so, it is possible that commercial organisations will exploit this technology by the large scale culturing of bacterial cells carrying recombinant vector molecules. It may be that, because of its photosynthetic abilities and metabolic plasticity, Rps.capsulata will be found to be a more economically attractive host cell, for large scale production, than e.g. E.coli. This potential may provide further impetus for the development of a Rps.capsulata/plasmid vector cloning system.

Other techniques, that have been recently developed, permit the determination of nucleotide sequences (Maxam and Gilbert, 1977; Sanger et al, 1977b). Such techniques

may be profitably applied to the study of the SP108 β -lactamase. As previously stated, the primary structure of β -lactamases, which may be derived from nucleotide sequence information, is relevant to several areas of enzymological research. Furthermore, such studies may be used to elucidate the structure of control regions adjacent to the structural gene, and indicate the presence of a polypeptide leader sequence. Thus, this type of study may also have implications for the possible processing and/or secretion of this enzyme.

A successful application of this technique may therefore permit us to take heed of Sir Arthur's advice.

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